

**A NOVEL ANTI-CD3/CD28 BEAD-BASED PHA/FEEDER-CELL-FREE HUMAN
IMMUNODEFICIENCY VIRUS-1 OUTGROWTH ASSAY**

by
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ABSTRACT

One of the major barriers to HIV-1 eradication is the presence of the latent reservoir, which is established very early in infection. This reservoir is widely dispersed throughout lymphoid tissue in the body. Resting latently infected CD4⁺ T cells are the best established cell type in the reservoir, and a number of clinical trials are currently investigating the effects of latency reversal agents on these cells. In order to determine the efficacy of such compounds, assays that measure the frequency of latently infected cells are of extreme importance. The gold standard quantitative viral outgrowth assay (QVOA) relies on mitogen phytohemagglutinin (PHA) in the presence of irradiated allogeneic PBMC (feeders) to achieve a global state of resting CD4⁺ T cell activation, followed by addition of PHA-stimulated CD4⁺ lymphoblasts at two time points for viral expansion. The need for addition of feeders followed by a step to remove PHA makes this assay costly and labor intensive. To address this issue, we developed an assay that does not require feeders and is less labor intensive. This new assay utilizes anti-CD3 and anti-CD28-coated microbeads for a more physiological activation of T cells. A side-by-side comparison of this assay to the conventional QVOA suggests that the sensitivity of the two techniques is similar. Additionally, stimulation with anti-CD3/CD28 coated microbeads resulted in faster activation of resting CD4⁺ T cells as determined from the percentage of cells expressing appropriate activation markers. The results indicate that this new assay could represent a sensitive, less expensive approach to quantifying the frequency of latently infected cells, which would have major implications for the HIV-1 cure agenda.

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CHAPTER 1 - INTRODUCTION

Historical Perspective

The June 5, 1981 edition of the Morbidity and Mortality Weekly Report (MMWR) from the Center for Disease Control and Prevention (CDC) deserves a special place in the history of immunology. One of the cases in the Epidemiologic Notes and Reports section covered a period spanning late fall of 1980 and early spring of 1981, whereas three different hospitals in the Greater Los Angeles Area treated five new cases of young men, all self-identified as homosexuals, for biopsy-confirmed *Pneumocystis carinii*. The latter is a rather rare condition in the United States and is primarily associated with severe immunosuppression. The condition of two of the patients deteriorated quickly and both eventually died a month after being hospitalized despite being put on standard treatment. The patients had no prior contacts with each other and three of them had significantly decreased white blood cell counts. “*All of the above observations suggest the possibility of a cellular-immune dysfunction related to a common exposure that predisposes individuals to opportunistic infections such as pneumocystosis...*” (Disease Control (CDC), 1981). This report was later recognized as the first report of the acquired immune deficiency syndrome (AIDS) in the United States (Disease Control & (CDC), 2001).

After subsequent epidemiologic observations suggested that AIDS was linked to an infectious pathogen and transmitted through sexual intercourse as well as contaminated blood, numerous research groups around the world embarked on a search of the primary etiologic agent of the disease. It was not until 1983 that Francoise Barre-

Sinoussi and Luc Montaigner from the Institute Pasteur in Paris were able to isolate the etiologic agent, a T-lymphotropic retrovirus, from the lymph node of a person with generalized lymphadenopathy of an unknown origin (Barré-Sinoussi et al., 1983). This finding was confirmed a year later by Robert Gallo, who demonstrated the connection between the infection and virus-specific immune responses in patients in addition to among the people at risk (Gallo et al., 1984; Popovic, Sarngadharan, Read, & Gallo, 1984; Sarngadharan, Popovic, Bruch, Schupbach, & Gallo, 1984). Two years later, in 1986, at around the same time that the latter AIDS-associated retrovirus was given a name of human immunodeficiency virus (HIV-1, (Coffin et al., 1986)), a related, but immunologically distinct virus was discovered in West Africa and termed HIV-2 (Clavel et al., 1986). While HIV-2 remained largely confined to its geographic area of origin, namely West Africa, HIV-1 has become a pandemic affecting millions of people around the world (Nyamweya et al., 2013).

HIV Epidemiology

According to the most recent statistics from the World Health Organization (WHO) and the CDC, there are currently 37 million people infected with HIV-1 worldwide and more than a million in the United State alone (“CDC - Statistics Overview - Statistics Center - HIV/AIDS”, “WHO | HIV/AIDS”). Every day there are 5,600 new infections in the world (“amfAR :: Statistics: Worldwide :: The Foundation for AIDS Research :: HIV / AIDS Research”). Sub-Saharan Africa, the region hit the hardest by the HIV/AIDS pandemic, has nearly 25 million people living with the infection alone, which is approximately two-thirds of all HIV-1 infected people (“Overview: HIV/AIDS - WHO

| Regional Office for Africa”). In the past decade, the overall trends in the global epidemiology of HIV have seen a dramatic change due to the increased number of people from low- and middle-income countries having access to the antiretroviral therapy (“WHO | Global update on HIV treatment 2013: Results, impact and opportunities,”). In fact, as a result of people living longer due to the success of the combined antiretroviral therapy (cART), the global prevalence of HIV has increased over the years, while the incidence has decreased (“Fact sheet 2015 | UNAIDS,”). However, despite the success in the global fight against HIV/AIDS, the latter is still a major contributor to the global burden of disease. In 2010 alone, HIV was found to be the leading cause of disability-adjusted life years for people aged 30-44 years worldwide, in addition to being the fifth leading cause for all ages (Ortblad, Lozano, & Murray, 2013).

HIV-1 Infection and Pathogenesis

HIV-1 is a lentivirus belonging to the family *Retroviridae* in the subfamily of *Orthoretrovirinae* (Fanales-Belasio, Raimondo, Suligoi, & Buttò, 2010). The virion itself consists of a genome, which is made of 2 single strands of positive-sense RNA containing 9 genes encoding 15 proteins (Figure 1) (Frankel & Young, 1998). The internal viral RNA is first protected by capsid proteins, and further covered with the remaining critical enzymes including reverse transcriptase, integrase, and protease by an outer membrane. The latter has as part of its structural architecture envelope glycoproteins, gp120/gp41 that facilitates binding and infection of the target cells through the interaction with the CD4 receptor and chemokine co-receptors.

CD4⁺ T cells and macrophages, which are the primary coordinators of the adaptive and innate immune systems, are the major targets for HIV. The transmission of the virus occurs predominantly through a sexual contact, and less common through other modes of exposure such as mechanical (needlesticks), breastfeeding, or blood transfusions. In the event of a sexual transmission, the virus or infected cells first penetrate the epithelium and find their way to the lamina propria where mucosal CD4⁺ T cells and macrophages are exposed to the virus via interactions with the dendritic cells (Pantaleo & Fauci, 1996). Afterward, the infected cells migrate to local lymph nodes where the virus is spread to local cells, ultimately resulting in the widespread systemic infection. As noted earlier, CD4⁺ T cells and macrophages are the primary targets for infections. This is explained by their expression of major receptors and co-receptors required for effective infection and dissemination of the virus, namely CD4 and CCR5 or CXCR4.

HIV replication inside the target cell generally involves reverse transcription of RNA to DNA followed by the nuclear transport and integration of viral DNA in the host genome. If successful, the proviral DNA will then undergo transcription into mRNA followed by translation, and assembly and release of infectious virions (Figure 2). Following the initial peak in viremia, HIV-specific humoral and cell-mediated responses can be detected by various methods. Anti-HIV antibodies as well as cytotoxic T lymphocytes (CTLs) are detected early in the infection and are responsible for the initial drop in viremia (Figure 3). A majority of the patients who are not receiving any medication usually die within ten years after acquiring the infection, most commonly due to opportunistic infections associated with the development of the immunodeficiency

(Deeks & Walker, 2007). Moreover, at about the same time as the epidemic took off, reports started appearing in the literature about individuals who were capable of sustaining a durable control over the virus. These individuals were positive by regular HIV antibody tests; however, they lacked any detectable virus, meaning their viral loads were consistently below 50 RNA copies HIV1 per milliliter of blood. They are now commonly referred to as elite controllers, or long-term non-progressors, constituting less than 1% of the HIV-infected population (O'Connell, Bailey, & Blankson, 2009; Okulicz & Lambotte, 2011). Interestingly enough, despite the lack detectable viremia, such individuals have been reported to be at a higher risk of developing atherosclerosis when compared to HIV-negative patients, suggesting factors other than cART are responsible for elevated risk of cardiovascular risk in HIV-infected individuals (Gandhi, Sax, & Grinspoon, 2012). Moreover, even among individuals capable of controlling the infection, a number of studies found a loss of control over time (Okulicz et al., 2009) further showing the importance of finding a cure.

The Challenge

The advent of combination antiretroviral therapy (cART) in the mid-90s has dramatically increased the longevity while simultaneously reducing the mortality and morbidity commonly associated with HIV-1 infections. In adherent patients, treatment with cART has been shown to reduce plasma viral load to below the limit of detection in clinical setting (50 copies HIV-1 RNA/mL of blood) (Sedaghat et al., 2009). However, despite all the advantages of this regiment, current treatment is not curative. Numerous studies have shown that patients on prolonged cART still harbor HIV-1 in its latent form,

fully replication-competent and integrated into the genomes of a small percentage of resting memory CD4⁺ T cells, which now serves as the best characterize cell reservoir for the HIV-1 in its latent form (Eisele & Siliciano, 2012). These cells are largely non-permissive of HIV-1 gene expression (Ruelas & Greene, 2013) and are of the long-lived lineage, as dictated by the biology of memory T cells, with a half-life of approximately 44 months (Blankson et al., 2000; Siliciano et al., 2003). Once the treatment is interrupted, internal signals allow for the viral genes to get reactivated and start the production of the infectious particles once again, requiring reinitiating the therapy (Harrigana, Whaley, & Montaner, 1999). It should also be noted that more often than not, the latent reservoir is composed of defective (i.e. replication-incompetent viruses) which arise due to error-prone enzyme reverse transcriptase. Such proviruses can occasionally revert to replication-competent variants, however it has been shown to be a relatively rare event (Ho et al., 2013; Sanchez, Xu, Chermann, & Hirsch, 1997; Simon-Loriere & Holmes, 2011). All in all, the profound stability of this HIV-1 reservoir prevents eradication with cART and is considered a major obstacle in fighting the pandemic (Deeks et al., 2012; Durand, Blankson, & Siliciano, 2012).

Currently, the most commonly circulated approach to eliminate the latent reservoir is to reverse latency via pharmacological methods so that infected cells can be eliminated either via cytopathic events or through the action of HIV-1 specific cytolytic T lymphocytes. This approach is also known as “shock-and-kill” (Nancie M Archin & Margolis, 2014; Deeks, 2012; Margolis, 2014). A number of clinical trials are currently investigating the effects of latency reversal agents on resting CD4⁺ T cells (Nancy M Archin et al., 2014; Rasmussen et al., 2014; Wei et al., 2014). In order to determine the

efficacy of such compounds, assays that measure the frequency of latently infected cells are of extreme importance (Bruner, Hosmane, & Siliciano, 2015). These assays work through the induction of global activation of CD4⁺ T cells in vitro in order to stimulate replication of latent virus. Methods used to achieve global activation include stimulation of resting CD4⁺ T cells with phytohemagglutinin (PHA) and irradiated allogeneic feeders (Finzi et al., 1997; Siliciano & Siliciano, 2005) or with immobilized anti-CD3 and anti-CD28 antibodies (Wong et al., 1997) or a bispecific CD3/CD8 antibody that simultaneously activates CD4⁺ T cells and depletes CD8⁺ T cells (Tremblay et al., 2000).

Currently, the PHA/feeders quantitative viral outgrowth assay (QVOA) is considered the gold standard for measuring the size of the latent reservoir (Siliciano & Siliciano, 2005). The assay induces activation of resting CD4⁺ T cells by the addition of PHA in the presence of a tenfold excess of irradiated allogeneic peripheral blood mononuclear cells (PBMCs). After overnight stimulation, the media containing PHA is removed and replaced with fresh media before CD4⁺ lymphoblasts are added to propagate the virus. The need for the addition of feeders followed by a PHA removal step makes this assay costly and labor intensive. The development of alternative assays which are less costly and labor intensive is a major priority for the HIV-1 cure agenda (Deeks et al., 2012).

The sensitivity of the PHA/feeders assay has not been directly compared to that of any of the antibody-based assays to the best of our knowledge. The antibody-based assays do not require the addition of feeders and therefore are less labor intensive. The first antibody-based assays used either immobilized anti-CD3 and anti-CD28 antibodies (Wong et al., 1997) or a soluble CD3/CD8 bispecific antibody. The two assays were

shown to have roughly the same sensitivity in a head to head comparison (Tremblay et al., 2000). A recent study has shown that anti-CD3/CD28-coated magnetic beads are more effective at stimulating primary CD4⁺ T cell growth than soluble anti-CD3 antibodies (Li & Kurlander, 2010). As such, we hypothesized that the more potent global stimulation and expansion of resting CD4⁺ T cells induced by anti-CD3/CD28-coated microbeads would lead to better outgrowth of latent virus. We developed a viral outgrowth assay with these beads and performed a direct comparison of the sensitivity of this new assay to that of the standard PHA/feeders assay.

Figure 1. Representation of the HIV-1 virion

A mature HIV-1 virion is composed of two copies of positive double-stranded RNA in addition to several proteins and enzymes that are essential for the infection and replication of the virus in the target cell.

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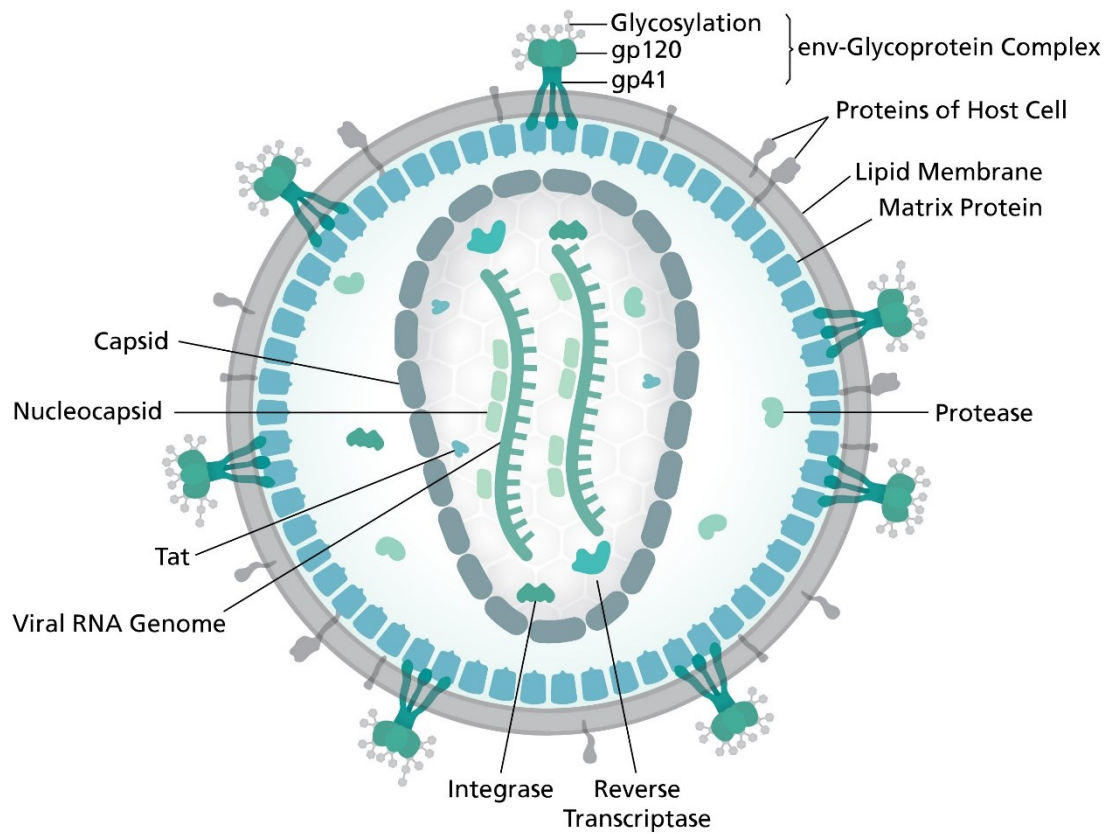


Figure 2. HIV-1 Replication

Schematic overview of the infection of an activated CD4⁺ T lymphocyte or macrophage by HIV. (A) Fusion and entry. (B) Reverse transcription. (C) Nuclear import and viral integration. (D) Proviral transcription and mRNA export. (E) Viral RNA translation and polyprotein assembly. (F) Viral assembly and release

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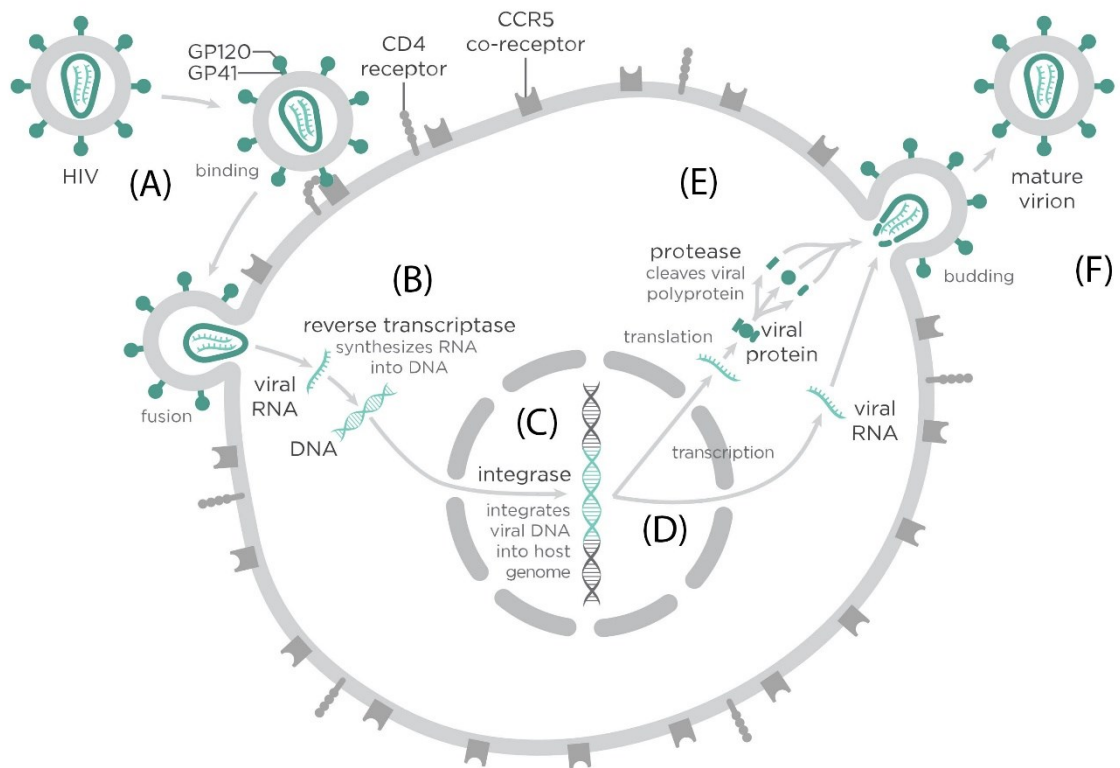
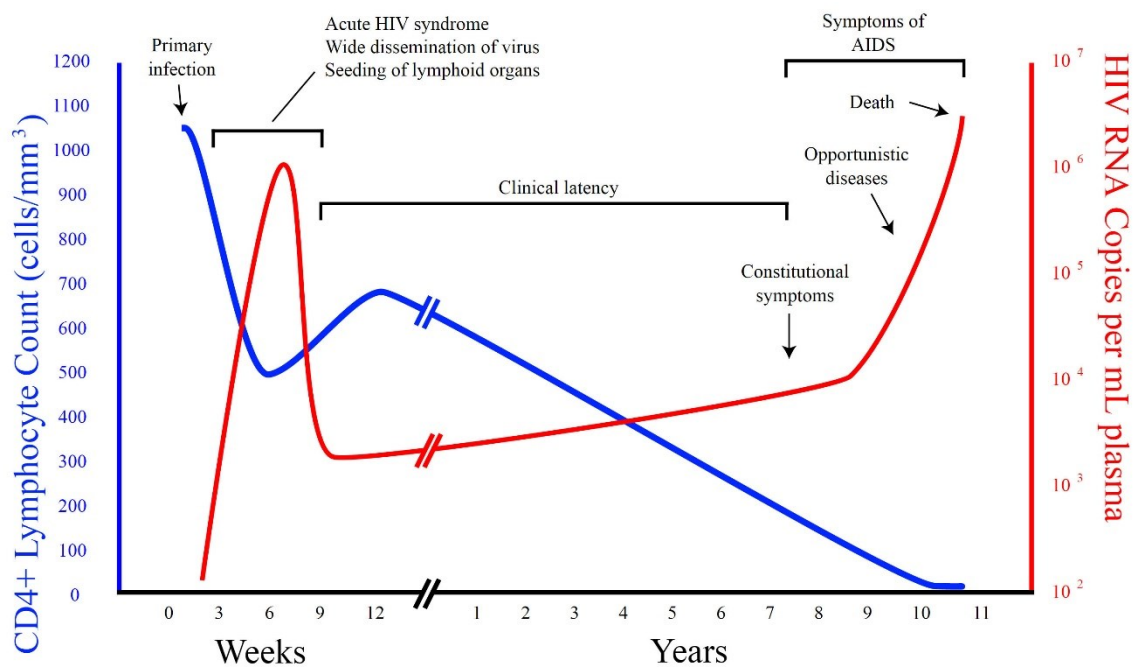


Figure 3. Natural History of HIV Infection in Untreated Individual

The first several weeks are characterized by acute flu-like symptoms with high viral titers in the blood. A restoration in CD4⁺ T cell count is observed after the activation of the adaptive immune system. Clinical latency, a largely asymptomatic phase, follows.

Opportunistic infections develop as the CD4⁺ T cell count falls (symptomatic phase). The patient is said to have AIDS when the CD4⁺ T cell count falls below 200 cell/ul

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CHAPTER 2 - METHODOLOGY

Patient Cohort

A total of thirteen HIV-1 infected individuals were enrolled in this study. Among them, three patients were elite suppressors with undetectable viral load. The remaining ten HIV-1 infected patients were on suppressive cART for more than a year when enrolled in this study. The clinical characteristics of these patients are presented in Table 1. The study was approved by the Johns Hopkins Institutional Review Board. All patients and HIV-1 negative donors provided written consent before participating in this study.

Isolation of Resting CD4⁺ T Cells

Peripheral blood was obtained from HIV-1-infected patients in a 30 ml syringe preloaded with 4 ml of acid-citrate-dextrose solution [ACD, Sigma-Aldrich, C3821] as an anticoagulant or a 60 ml syringe with 8 ml of ACD. We also collected blood from healthy donors. The latter was performed by using a 60 ml syringe with 700 μ l of heparin (Appendix – Materials 2) as an anticoagulant. PBMCs were subsequently isolated by Ficoll gradient centrifugation (Appendix - Methods 2). CD4⁺ T cells were isolated from bulk PBMCs by negative selection (Appendix – Methods 3). Afterward, we performed isolation of resting CD4⁺ T lymphocytes (Appendix – Methods 4). Purified resting CD4⁺ T cells were resuspended in STCM (Appendix – Materials 4).

Viral Outgrowth Assays

Resting CD4⁺ T cells from each patient were divided into 2 aliquots – 1 aliquot was used for the CD3/CD28 assay, the other for the PHA/feeders QVOA. We initially started with 25 to 30 replicates of 2×10^5 resting CD4⁺ T cells. For the PHA/feeders assay, 2×10^5 cells were cultured with 2.5 million irradiated PBMCs (Appendix – Methods 5) in each well of a 24-well plate in 2ml of STCM (Appendix – Materials 4).

For the alternate assay, 2×10^5 cells were cultured in each well of a 96-well plate in 200 ul of STCM described above and 5 ul of anti-CD3/CD28-coated microbeads (ThermoFisher Scientific, 11161D).

CD4⁺ T cells were stimulated for 18 hours in both assays. Afterward, for the PHA/feeders assay, the PHA was washed off and 0.5 to 1×10^6 lymphoblasts were added to each well (Appendix – Methods 6 and 7). For the alternate assay, the cells and beads were resuspended and transferred to 48-well plate wells with 0.5 to 1 million lymphoblasts (Appendix – Methods 7) per well.

A new round of 0.5 to 1 million lymphoblasts were added to each well on day 9 for both assays.

In patients where virus was not obtained using multiple replicates of 2×10^5 CD4⁺ T cells, the assay was repeated with 20 to 30 replicates of 1×10^6 resting CD4⁺ T cells.

For the PHA/feeders assay, 1×10^6 cells were cultured with 10 million irradiated feeders in each well of a 6-well plate in 8ml of STCM.

For the anti-CD3/CD28 beads-based assay, 1×10^6 cells were cultured in each well of a 48-well plate in 1ml of STCM and 25 ul of anti-CD3/CD28-coated microbeads.

CD4⁺ T cells were stimulated for 18 hours in both assays. For the PHA/feeders assay, the PHA was removed and 2 to 4 million lymphoblasts were added to each well.

For the alternate assay, the cells and beads were resuspended and transferred to 12-well plate wells with 2 to 4 million lymphoblasts added per well.

A new round of 2 to 4 million blasts were added to each well on day 9 for both assays.

In patients where virus was not obtained using replicates of 1×10^6 CD4⁺ T cells, the anti-CD3/CD28 microbead assay was repeated with 30 to 45 replicates of 2×10^5 cells with the addition of a spinoculation step during the first round of lymphoblast addition. In brief, 2×10^5 blasts in 20 μ l STCM was added to each well of a 96-well plate. It was then spinoculated at 1200 x G for 2 hours at 37°C. Cells from each well were then transferred to a 48 well plate and 8×10^5 additional blasts were added to each well. The volume was adjusted to 1 ml with the addition of 700 μ l of STCM.

Lymphoblasts from the same healthy donors were always used to amplify virus from the two assays for each patient in order to minimize the number of variables in the comparison

Supernatants from each well were tested for HIV-1 p24 protein by ELISA (Appendix – Methods 9) on day 12 and repeated on day 19 for the negative wells. For both the anti-CD3/CD28 bead-based assay and the standard PHA/feeders assay, incubation was done at 37°C under 5% CO₂.

The overall assay setup is presented in Figure 4.

Flow Cytometric Analysis of Activated Resting CD4⁺ T Cells

To compare the activation levels achieved in the 2 donors, CD4⁺ T cells were obtained from three HLA-A2-positive healthy donors. 2×10^5 cells were plated in triplicates for each time point and stimulated as described above for the anti-CD3/CD28 and PHA/feeders assays. The feeders were obtained from a HLA-A2-negative HIV-uninfected donor so that we could easily distinguish between the feeders and the purified resting CD4⁺ T cells using an antibody specific for HLA-A2. After 18 hours of incubation, the cells from the standard assay were stained with CD3-PacBlue (BD), CD8-APC-H7 (BD), CD4-PE (BD), HLA-A2-PerCP-Cy5.5 (BioLegend), CD25-FITC (BD), HLA-DR-APC (BD), and CD69-BV605 (BD). For the CD3/CD28 assay, the beads were removed prior to staining. Cells were fixed and permeabilized with Cytoperm/Cytofix Kit (BD). Stained cells were analyzed by flow cytometry on a FACSCanto II (BD). The procedure was repeated on day 2 and 3 (Appendix – Methods 10, 11).

Data Analysis

Flow cytometry data was analyzed using FlowJo v10.0.7. All statistical analysis was performed in GraphPad Prism v6.01. A Wilcoxon matched-pairs signed rank test was used for statistical analysis. Frequencies of infected cells (infectious units per million wells [IUPM]) were based on the percentage of positive replicates and were calculated as previously described (Siliciano & Siliciano, 2005).

Table 1. Clinical characteristics of the patients studied

Subject	Current CD4⁺ T cell count	Nadir CD4⁺ T cell count	Time on suppressive regiment	Current regiment
PT 8	424	18	8 years	3TC, RAL EFV
PT 10	1109	NA	12 years	3TC, ABC, DTG
PT 11	1032	177	8 years	TDF, FTC, DRV/r
PT 12	860	494	3 years	3TC, RAL EFV
PT 14	646	12	4 years	DRV/r, DTG
PT 16	921	203	5 years	3TC, ABC, DTG
PT 21	541	388	3 years	FTC, TDF, RPV
PT 40	705	NA	4 years	FTC, TDF, EFV
PT 42	1140	NA	4 years	MVC, DRV/r, RAL
PT 45	402	19	7 years	DTG, DRV/r
ES 31	NA	NA	NA	NA
ES 23	NA	NA	NA	NA
ES 46	NA	NA	NA	NA

3TC: lamivudine, ABC: abacavir, FTC: emtricitabine, TDF: tenofovir, DTG: dolutegravir, EFV: efavirenz, RPV: rilpivavine, RAL: raltegravir, MVC: maraviroc, DRV/c: cobicistat boosted darunavir, DRV/r: ritonavir boosted darunavir

NA: Not available

Figure 4. The standard PHA/feeders and anti-CD3/CD28 viral outgrowth assays

Schematic representation of the side-by-side experimental setup using PHA and γ -irradiated allogeneic PBMCs for the PHA/feeders standard and anti-CD3/CD28 microbeads for the alternative anti-CD3/CD28-beads-based viral outgrowth assay.

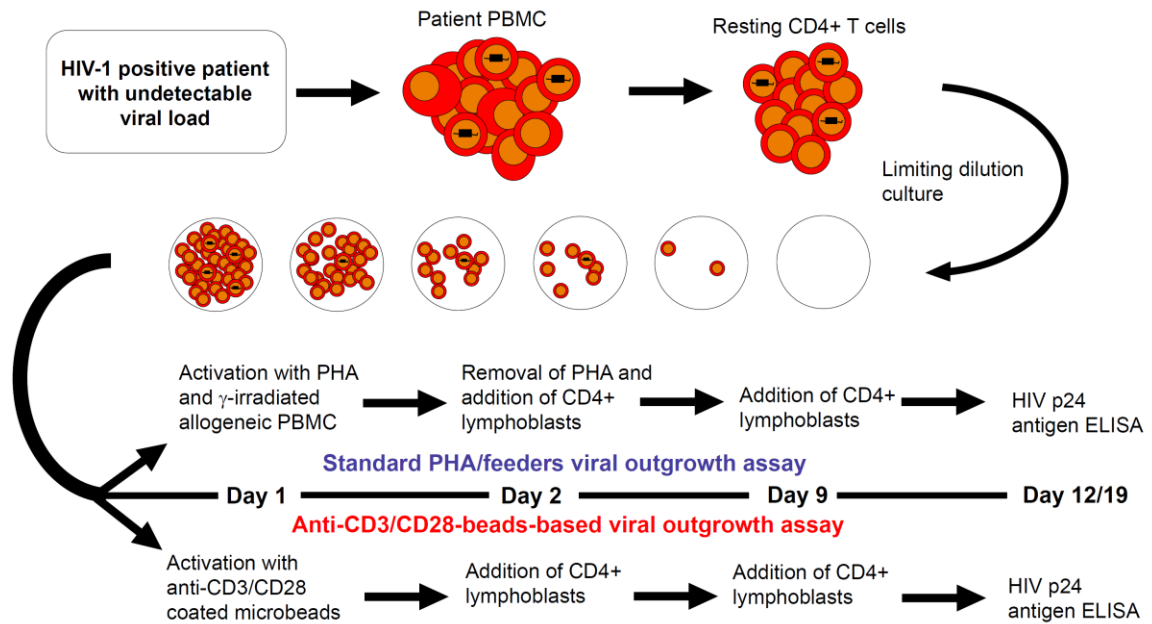


Figure 1. The standard and alternative viral outgrowth assays. Schematic representation of the side-by-side experimental setup using PHA and γ -irradiated allogeneic PMBC for the standard assay and anti-CD3/CD28 microbeads for the alternative assay.

Courtesy of Dr. Robert Siliciano

CHAPTER 3 - RESULTS

Stimulation with anti-CD3/CD28-coated microbeads results in faster activation of resting CD4⁺ T cells

We first compared the level of immune activation achieved with the traditional PHA/feeders assay to that achieved with anti-CD3/CD28-coated microbeads using resting CD4⁺ T cells from 3 healthy donors. Analysis of T cell activation by flow cytometry showed that stimulation with microbeads results in faster activation of resting CD4⁺ T cells as determined from the percentage of cells expressing very early (CD69), early (CD25), and late (HLA-DR) activation markers (Figure 5) (Reddy, Eirikis, Davis, Davis, & Prabhakar, 2004). Notably, on day 1 post stimulation, between 80-100% of cells stimulated with anti-CD3/CD28 beads expressed CD69 and CD25, while for the PHA/feeders activated cells the values ranged from 15-50%. Figure 6 shows the overall gating strategy.

The sensitivity of the anti-CD3/CD28 bead-based assay is similar to that of the PHA/feeders assay

We originally plated 25 to 30 replicates of 2×10^5 resting CD4⁺ T cells per well for both assays. The median percentage of positive wells was 15% (range from 0% to 40%) for the standard PHA/feeders assay and 10% (range from 0% to 68%) for the bead-based assay. No virus was isolated from the PHA/feeders assay in 1 of 10 patients tested (PT40) whereas no virus was obtained with the bead-based assay in 4 out of 10 patients (PT8, PT14, PT21, and PT40) (Figure 7A).

To improve the sensitivity of both assays, we repeated the experiment with more patient cells using 20 to 30 replicates of 1×10^6 resting CD4⁺ T cells from the four patients from whom no virus was obtained in one or both assays (PT8, PT14, PT21, and PT40). While virus was amplified in all 4 patients with the PHA/feeders assay, no virus was amplified from PT21 and PT40 with the bead-based assay (Figure 7B). The median percentage of positive wells was 19% (range from 13% to 40%) for the PHA/feeders assay and 5% (range from 0% to 10%) for the bead-based assay. IUPMs were calculated using the 2×10^5 cell replicates for the 6 patients for whom virus was amplified with both assays. The 1×10^6 cell replicates were used to determine the IUPMs for the other 4 patients. No significant difference was observed between the frequency of latently infected resting CD4⁺ T cells as measured by the PHA/feeders assay compared to the bead-based assay (Figure 7C, Wilcoxon matched-pairs signed rank test, $p = 0.5469$). Moreover, there was a significant correlation between the frequency of latently infected cells measured in the two different assays (Figure 7D, Pearson's correlation coefficient, $r = 0.8520$, $p = 0.0017$). Raw data for the p24 assays is provided in Table 2 through Table 15. We were not able to detect any virus following the spinoculation procedure (data shown in Table 16 through Table 18). Moreover, the anti-CD3/CD28 assay was not able to detect virus from the elite controllers, while the PHA/feeders detected virus in one of the patients (data shown in Table 19 through Table 21).

Figure 5. Stimulation of lymphocytes with anti-CD3/CD28 microbeads exhibits faster activation of resting CD4⁺ T cells as compared to the PHA/feeders standard viral outgrowth assay

Resting CD4⁺ T cells from three healthy donors were activated with standard PHA/feeders or with anti-CD3/CD28 microbeads and analyzed for the expression of markers of cellular activation by flow cytometry. (A) CD69 Expression. (B) CD25 Expression. (C) HLA-DR Expression.

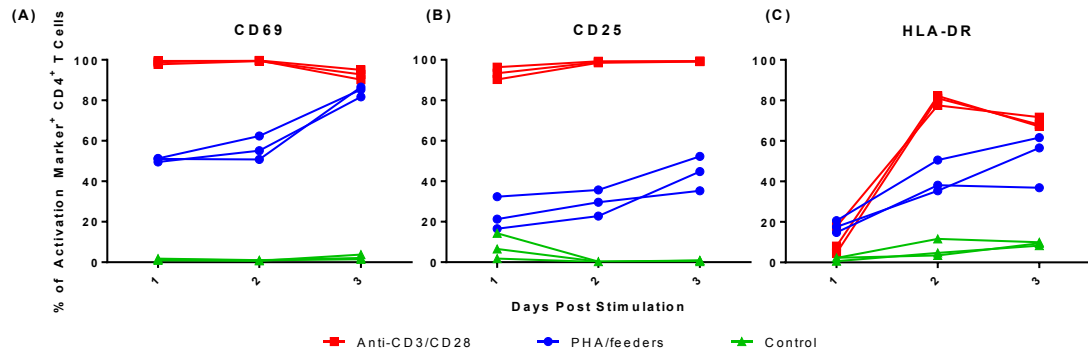


Figure 6. Representative FACS gating strategy for cell populations expressing different activation markers

Initial lymphocyte populations gated based on forward and side scatter properties. HLA-A2-negative donor served as the control for distinguishing between feeders and cell of interest.

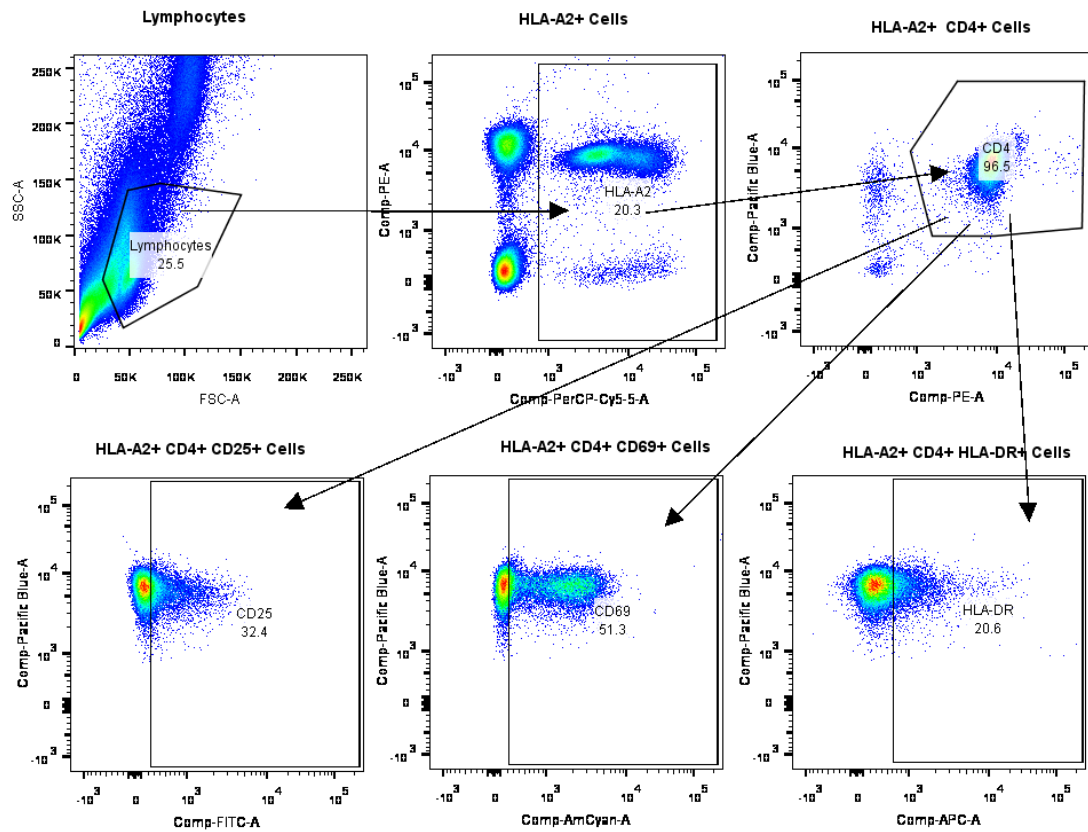


Figure 7. The standard PHA/feeders and anti-CD3/CD28-beads-based viral outgrowth assays yield comparable results

(A) For both assays, 2×10^5 cells were plated side-by-side in appropriate multi-well culture plates. The percentage of wells positive for the p24 antigen is shown. N is the number of wells tested for each assay. (B) For both assays, 1×10^6 cells were plated side-by-side in appropriate multi-well culture plates. The percentage of wells positive for the p24 antigen is shown. N is the number of wells tested for each assay. (C) Statistical comparison of the infectious units per million cells (IUPM) values using the 2 assays by Wilcoxon matched-pairs signed rank test. Virus was not detected in 2 patients with the anti-CD3/CD28 bead-based assay (PT21 and PT40) and the upper limit of the IUPM for both patients is shown in open red circles. (D) The correlation of infectious units per million cells (IUPM) values using the standard PHA/feeders and anti-CD3/CD28 assays (Pearson's correlation coefficient, r). PT21 and PT40 are shown as open circle. PT42 and PT10 are shown as a square.

Figure 7 (cont.)

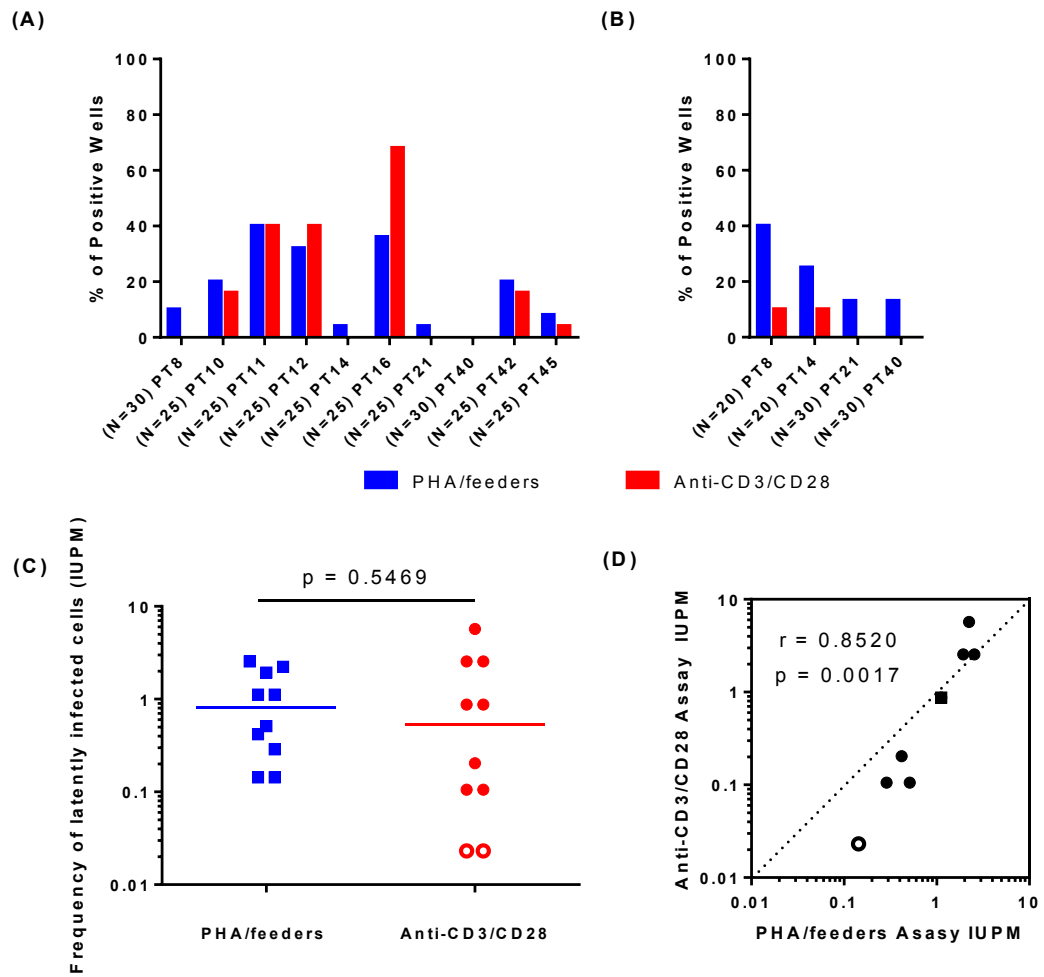


Table 2. Setup of the p24 ELISA Assay for PT8 (2x10⁵ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight denotes a positive well. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	2.0231	3.5955	0.0542	0.0639	0.0583	0.0752	0.0648	0.0567	0.0598	0	0	-0.0029
B	1.1782	0.0549	3.4752	0.1853	0.0972	0.0548	0.0652	0.0627	0.0621	0.0001	0.0001	-0.0025
C	0.5724	0.0541	0.0683	0.2118	0.0754	0.0562	0.0526	0.0537	0.0616	0.0003	0.0002	-0.0024
D	0.3454	0.0537	0.0527	0.2263	0.0529	0.059	0.0539	0.0568	0.0548	0.0002	0.0001	-0.0025
E	0.2025	0.0536	0.077	0.0535	0.0521	0.0563	0.0592	0.0546	0.0904	0.0002	0	-0.0026
F	0.1314	0.0543	0.0569	0.0553	0.0608	0.0572	0.057	0.0533	0.0599	0.0002	0.0001	-0.0027
G	0.0586	3.4822	0.0585	0.0537	0.0534	0.0558	0.0539	0.0587	0.0535	0.0001	0.0001	-0.0026
H	0.0509	0.0608	0.0635	0.0948	0.0556	0.0751	0.0562	0.0541	0.0546	0.0001	0.0002	-0.0027

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N1	N9	N17	N25			
B	Standard 50pg/ml	C2	C10	C18	C26	N2	N10	N18	N26			
C	Standard 25pg/ml	C3	C11	C19	C27	N3	N11	N19	N27			
D	Standard 12.5pg/ml	C4	C12	C20	C28	N4	N12	N20	N28			
E	Standard 6.25pg/ml	C5	C13	C21	C29	N5	N13	N21	N29			
F	Standard 3.125pg/ml	C6	C14	C22	C30	N6	N14	N22	N30			
G	STCM Control	C7	C15	C23	G1	N7	N15	N23	STCM Control			
H	Substrate Blank	C8	C16	C24	G2	N8	N16	N24	STCM Control			

Table 3. Setup of the p24 ELISA Assay for PT10 (2x10⁵ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight - positive well. Red highlight – well is not applicable for further analysis. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.5562	3.5892	3.5662	0.0496	0.0492	0.0558	0.0519	0.0489	0.0495	0	0.0001	-0.0024
B	0.846	0.0533	0.0541	0.0609	0.05	0.4846	0.055	0.0596	3.4967	0.0001	0.0001	-0.0021
C	0.3801	0.0487	0.048	0.0493	0.0491	0.0502	0.0544	0.049	0.0594	0.0002	0.0002	-0.0019
D	0.235	0.0501	3.2415	0.0496	0.0496	0.0496	0.0518	0.0481	0.0511	0.0001	0.0001	-0.002
E	0.1253	0.0486	0.0478	0.0482	0.0663	0.0487	0.0514	0.0489	0.0589	0.0001	0.0001	-0.0022
F	0.0925	3.4824	0.0493	0.0494	0.0498	0.0511	0.0493	0.05	0.0619	0	0.0001	-0.002
G	0.05	0.0522	0.0492	0.0506	0.0499	0.0513	0.0552	0.0538	0.0542	0.0001	0.0001	-0.002
H	0.0527	0.0502	0.0515	3.6266	0.0516	3.6891	3.3012	3.5604	0.0523	0.0001	0.0001	-0.0026

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N1	N9	N17	N25			
B	Standard 50pg/ml	C2	C10	C18	C26	N2	N10	N18	N26			
C	Standard 25pg/ml	C3	C11	C19	C27	N3	N11	N19	N27			
D	Standard 12.5pg/ml	C4	C12	C20	C28	N4	N12	N20	N28			
E	Standard 6.25pg/ml	C5	C13	C21	C29	N5	N13	N21	N29			
F	Standard 3.125pg/ml	C6	C14	C22	C30	N6	N14	N22	N30			
G	STCM Control	C7	C15	C23	G1	N7	N15	N23	STCM Control			
H	Substrate Blank	C8	C16	C24	G2	N8	N16	N24	STCM Control			

Table 4. Setup of the p24 ELISA Assay for PT11 (2x10⁵ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight denotes a positive well. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.9443	0.0527	3.6377	0.06	0.0549	3.6118	0.0533	0.0532	0.0001	0.0001	0	-0.0023
B	0.5982	0.0539	3.5156	3.4984	0.0556	3.5114	0.0563	0.0523	0.0001	0.0001	0.0001	-0.0021
C	0.3054	3.5039	0.0527	0.0521	0.0606	0.0651	0.0602	0.0527	0.0002	0.0002	0.0002	-0.0019
D	0.2121	0.0557	0.0544	1.061	3.5067	0.0588	3.5075	0.0582	0.0001	0.0002	0.0001	-0.002
E	0.149	0.0579	0.0548	0.0519	0.0527	0.0523	3.4323	0.0532	0.0001	0.0001	0.0001	-0.002
F	0.0938	0.0541	1.5274	0.0525	0.0535	0.0786	3.4986	0.0538	0.0001	0.0001	0.0001	-0.002
G	0.0607	0.0558	3.5241	3.5303	3.52	0.0521	0.0624	0.0533	0.0002	0.0001	0.0002	-0.0019
H	0.054	0.054	3.6709	3.6946	3.7251	3.6077	0.1895	0.0523	0.0002	0.0002	0.0001	-0.0023

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N8	N16	N24				
B	Standard 50pg/ml	C2	C10	C18	N1	N9	N17	N25				
C	Standard 25pg/ml	C3	C11	C19	N2	N10	N18	G1				
D	Standard 12.5pg/ml	C4	C12	C20	N3	N11	N19	G2				
E	Standard 6.25pg/ml	C5	C13	C21	N4	N12	N20	STCM Control				
F	Standard 3.125pg/ml	C6	C14	C22	N5	N13	N21	STCM Control				
G	STCM Control	C7	C15	C23	N6	N14	N22	STCM Control				
H	Substrate Blank	C8	C16	C24	N7	N15	N23	STCM Control				

Table 5. Setup of the p24 ELISA Assay for PT12 (2x10⁵ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight denotes a positive well. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.9925	3.6293	0.0724	0.0545	0.0663	0.3859	0.0615	3.6229	0	0	0	-0.0024
B	0.5501	0.0583	3.5181	0.0944	0.1227	1.1035	0.0683	0.0626	0	0	0	-0.0021
C	0.2992	0.0729	0.057	0.0666	3.5145	3.5614	0.0598	0.0572	0.0001	0.0001	0.0002	-0.0019
D	0.1813	0.0749	0.0568	0.0607	0.0619	3.5462	0.0608	0.056	0.0001	0.0001	0.0001	-0.0021
E	0.1222	0.0667	0.0613	0.063	0.0593	3.5794	0.08	0.059	0	0.0001	0	-0.0021
F	0.0951	3.5064	1.1001	0.058	0.0953	3.5409	0.0694	0.0548	0.0001	0	0.0001	-0.0019
G	0.0592	0.3144	3.5486	0.2158	0.0573	0.0676	3.4879	0.0575	0.0001	0.0002	0	-0.002
H	0.0508	0.058	0.0558	0.1242	0.0601	0.0562	0.0569	0.0557	0.0001	0.0001	0.0001	-0.0024

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N8	N16	N24				
B	Standard 50pg/ml	C2	C10	C18	N1	N9	N17	N25				
C	Standard 25pg/ml	C3	C11	C19	N2	N10	N18	G1				
D	Standard 12.5pg/ml	C4	C12	C20	N3	N11	N19	G2				
E	Standard 6.25pg/ml	C5	C13	C21	N4	N12	N20	STCM Control				
F	Standard 3.125pg/ml	C6	C14	C22	N5	N13	N21	STCM Control				
G	STCM Control	C7	C15	C23	N6	N14	N22	STCM Control				
H	Substrate Blank	C8	C16	C24	N7	N15	N23	STCM Control				

Table 6. Setup of the p24 ELISA Assay for PT14 (2x10⁵ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight denotes a positive well. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.8885	0.0557	0.0548	0.0546	0.0558	0.0547	0.0605	0.0602	0.0001	0.0001	0	-0.0024
B	0.492	0.0558	0.0559	0.0642	0.0573	0.0573	0.077	0.0607	0.0001	0.0001	0.0001	-0.0021
C	0.3126	0.0554	0.0593	0.0597	0.0634	0.0569	0.0575	0.0549	0.0001	0.0002	0.0002	-0.0018
D	0.1883	0.0567	0.056	0.0571	0.0568	0.0559	0.0568	0.0562	0.0001	0.0001	0.0001	-0.002
E	0.1412	0.0705	0.0791	0.055	0.0565	0.0579	0.0591	0.0594	0.0001	0.0001	0.0001	-0.0021
F	0.0937	0.0728	0.2462	0.0577	0.0559	0.0585	0.0589	0.0568	0	0.0001	0.0001	-0.0019
G	0.0631	0.0642	0.0702	0.0551	0.0564	0.0574	0.0579	0.0566	0.0001	0.0002	0.0001	-0.002
H	0.0495	0.0951	0.056	3.6738	0.0558	0.056	0.0604	0.0564	0.0002	0.0001	0.0001	-0.0024

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N8	N16	N24				
B	Standard 50pg/ml	C2	C10	C18	N1	N9	N17	N25				
C	Standard 25pg/ml	C3	C11	C19	N2	N10	N18	G1				
D	Standard 12.5pg/ml	C4	C12	C20	N3	N11	N19	G2				
E	Standard 6.25pg/ml	C5	C13	C21	N4	N12	N20	STCM Control				
F	Standard 3.125pg/ml	C6	C14	C22	N5	N13	N21	STCM Control				
G	STCM Control	C7	C15	C23	N6	N14	N22	STCM Control				
H	Substrate Blank	C8	C16	C24	N7	N15	N23	STCM Control				

Table 7. Setup of the p24 ELISA Assay for PT16 (2x10⁵ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight - positive well. Red highlight – well is not applicable for further analysis. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.7812	0.0797	0.0574	0.0575	0.059	0.1414	3.5447	3.5984	3.5962	0	-0.0001	-0.003
B	1.1504	3.4965	0.6067	0.0553	0.0601	0.0899	3.4875	3.5022	0.0801	0	0.0001	-0.0026
C	0.6494	0.0608	0.0586	0.058	0.0668	0.3764	3.5279	0.0629	3.5083	0.0002	0.0002	-0.0024
D	0.3918	0.0617	3.5111	0.0949	0.096	3.5379	0.0648	3.496	1.8293	0.0001	0.0001	-0.0025
E	0.2185	0.0589	0.1186	0.0771	3.6094	1.0832	0.0606	3.3751	0.0919	0.0001	0	-0.0027
F	0.1356	0.0598	3.5413	0.0589	0.0581	0.0771	3.5201	0.0672	2.3526	0	0.0001	-0.0027
G	0.0584	0.0606	3.5563	3.5382	0.0585	0.0775	3.5421	1.075	0.058	0.0001	0	-0.0026
H	0.0525	3.6918	0.2906	3.6487	0.0578	1.9885	3.6712	3.6233	0.0593	0.0001	0.0001	-0.0028

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N1	N9	N17	N25			
B	Standard 50pg/ml	C2	C10	C18	C26	N2	N10	N18	N26			
C	Standard 25pg/ml	C3	C11	C19	C27	N3	N11	N19	N27			
D	Standard 12.5pg/ml	C4	C12	C20	C28	N4	N12	N20	N28			
E	Standard 6.25pg/ml	C5	C13	C21	C29	N5	N13	N21	N29			
F	Standard 3.125pg/ml	C6	C14	C22	C30	N6	N14	N22	N30			
G	STCM Control	C7	C15	C23	G1	N7	N15	N23	STCM Control			
H	Substrate Blank	C8	C16	C24	G2	N8	N16	N24	STCM Control			

Table 8. Setup of the p24 ELISA Assay for PT21 (2x10⁵ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight - positive well. Red highlight – well is not applicable for further analysis. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	2.3775	0.0619	0.0577	0.0616	0.0571	0.06	0.0586	0.0631	0.0591	0.0001	0.0001	-0.0024
B	1.4358	0.0566	3.4726	0.0688	0.0573	0.0613	0.0573	0.0644	0.069	0.0002	0.0001	-0.0021
C	0.7173	0.0595	0.0541	0.0571	0.0552	0.0559	0.0598	0.0552	0.0707	0.0002	0.0001	-0.0019
D	0.4007	0.0545	0.0574	0.0553	0.0541	0.0551	0.0548	0.0555	0.0593	0.0002	0.0003	-0.002
E	0.2481	0.0546	0.055	0.0569	0.0576	0.0557	0.1016	0.0553	0.0561	0.0001	0	-0.0021
F	0.1501	0.057	0.0741	0.0638	0.0554	0.0563	0.0557	0.0561	0.0767	0.0001	0.0001	-0.0021
G	0.056	0.075	0.0666	0.0588	0.056	0.0562	0.0571	0.0562	0.0566	0	0.0001	-0.0021
H	0.052	0.057	0.0586	0.0579	0.063	0.0575	0.0578	0.0562	0.0566	0.0001	0.0001	-0.0026

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N1	N9	N17	N25			
B	Standard 50pg/ml	C2	C10	C18	C26	N2	N10	N18	N26			
C	Standard 25pg/ml	C3	C11	C19	C27	N3	N11	N19	N27			
D	Standard 12.5pg/ml	C4	C12	C20	C28	N4	N12	N20	N28			
E	Standard 6.25pg/ml	C5	C13	C21	C29	N5	N13	N21	N29			
F	Standard 3.125pg/ml	C6	C14	C22	C30	N6	N14	N22	N30			
G	STCM Control	C7	C15	C23	G1	N7	N15	N23	STCM Control			
H	Substrate Blank	C8	C16	C24	G2	N8	N16	N24	STCM Control			

Table 9. Setup of the p24 ELISA Assay for PT40 (2x10⁵ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.9111	0.0492	0.0493	0.0541	0.0499	0.053	0.0521	0.0554	0.0516	0.0001	0.0001	-0.0026
B	1.0471	0.0493	0.0524	0.0486	0.0534	0.0595	0.058	0.05	0.0477	0.0001	0.0001	-0.0022
C	0.5826	0.0494	0.0485	0.0482	0.048	0.0472	0.0497	0.0472	0.0528	0.0003	0.0002	-0.002
D	0.2874	0.0482	0.0478	0.0477	0.0484	0.049	0.0501	0.0468	0.0476	0.0002	0.0001	-0.0021
E	0.188	0.0486	0.0476	0.0555	0.0535	0.0471	0.0469	0.0471	0.0483	0.0001	0.0001	-0.0023
F	0.1164	0.0499	0.0483	0.0486	0.0485	0.0481	0.0484	0.0473	0.0592	0.0001	0.0002	-0.0022
G	0.0496	0.049	0.0483	0.0518	0.0488	0.0473	0.0488	0.0468	0.0532	0.0002	0.0001	-0.0022
H	0.0483	0.0486	0.0487	0.0489	0.0505	0.0494	0.0488	0.0488	0.0485	0.0001	0.0001	-0.0025

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N1	N9	N17	N25			
B	Standard 50pg/ml	C2	C10	C18	C26	N2	N10	N18	N26			
C	Standard 25pg/ml	C3	C11	C19	C27	N3	N11	N19	N27			
D	Standard 12.5pg/ml	C4	C12	C20	C28	N4	N12	N20	N28			
E	Standard 6.25pg/ml	C5	C13	C21	C29	N5	N13	N21	N29			
F	Standard 3.125pg/ml	C6	C14	C22	C30	N6	N14	N22	N30			
G	STCM Control	C7	C15	C23	G1	N7	N15	N23	STCM Control			
H	Substrate Blank	C8	C16	C24	G2	N8	N16	N24	STCM Control			

Table 10. Setup of the p24 ELISA Assay for PT42 (2x10⁵ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight denotes a positive well. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.845	0.0925	0.0573	3.5789	0.0564	0.0605	0.0558	0.0571	0	0	0	-0.0026
B	0.6741	0.0576	1.429	0.0604	3.521	0.0593	0.0577	0.0562	0.0001	0.0001	0	-0.0023
C	0.3622	0.0606	3.4824	0.0616	0.23	0.1441	0.062	0.0575	0.0001	0.0002	0.0002	-0.0019
D	0.2263	0.0608	0.0605	0.0573	0.0862	0.0711	0.0556	0.0559	0.0001	0.0001	0.0001	-0.0021
E	0.1436	0.0684	0.0661	0.0586	0.0631	0.0588	0.0668	0.0584	0	0	0	-0.0022
F	0.1012	0.0601	0.0567	0.0991	0.0583	0.0578	0.0596	0.0868	0.0001	0.0001	0.0001	-0.0022
G	0.0592	0.0594	0.0632	3.5707	0.0608	0.0647	0.0568	0.057	0.0001	0.0001	0.0001	-0.0022
H	0.0523	0.0564	3.6769	0.0567	0.0833	0.089	3.6733	0.0598	0.0002	0.0001	0.0001	-0.0025

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N8	N16	N24				
B	Standard 50pg/ml	C2	C10	C18	N1	N9	N17	N25				
C	Standard 25pg/ml	C3	C11	C19	N2	N10	N18	G1				
D	Standard 12.5pg/ml	C4	C12	C20	N3	N11	N19	G2				
E	Standard 6.25pg/ml	C5	C13	C21	N4	N12	N20	STCM Control				
F	Standard 3.125pg/ml	C6	C14	C22	N5	N13	N21	STCM Control				
G	STCM Control	C7	C15	C23	N6	N14	N22	STCM Control				
H	Substrate Blank	C8	C16	C24	N7	N15	N23	STCM Control				

Table 11. Setup of the p24 ELISA Assay for PT45 (2x10⁵ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight denotes a positive well. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.333	0.0563	0.0561	0.0575	0.0567	0.0584	0.0597	0.0661	0.0001	0.0001	0.0001	-0.0025
B	0.7931	0.0571	0.0566	3.5234	0.0569	0.0578	0.0655	0.0571	0.0001	0.0002	0.0001	-0.0022
C	0.4188	0.0567	0.057	3.5013	0.0643	0.0583	0.0596	0.0563	0.0002	0.0002	0.0002	-0.002
D	0.2388	0.06	0.0564	0.0614	0.0578	0.058	0.0713	0.0566	0.0001	0.0002	0.0002	-0.002
E	0.1617	0.0563	0.0582	0.0558	0.0562	0.0568	0.0565	0.055	0.0001	0.0001	0	-0.0022
F	0.1084	0.0587	0.0562	0.0561	0.0598	3.5506	0.0564	0.0565	0.0001	0.0001	0.0001	-0.0021
G	0.0579	0.057	0.0566	0.0574	0.0624	0.0565	0.065	0.0597	0.0001	0.0001	0.0002	-0.0021
H	0.0509	0.059	0.0583	0.0576	0.0594	0.058	0.0586	0.0606	0.0002	0.0002	0.0001	-0.0024

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N8	N16	N24				
B	Standard 50pg/ml	C2	C10	C18	N1	N9	N17	N25				
C	Standard 25pg/ml	C3	C11	C19	N2	N10	N18	G1				
D	Standard 12.5pg/ml	C4	C12	C20	N3	N11	N19	G2				
E	Standard 6.25pg/ml	C5	C13	C21	N4	N12	N20	STCM Control				
F	Standard 3.125pg/ ml	C6	C14	C22	N5	N13	N21	STCM Control				
G	STCM Control	C7	C15	C23	N6	N14	N22	STCM Control				
H	Substrate Blank	C8	C16	C24	N7	N15	N23	STCM Control				

Table 12. Setup of the p24 ELISA Assay for PT8 (1x10⁶ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight - positive well. Red highlight – well is not applicable for further analysis. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.4009	0.0891	3.5147	3.5707	3.4757	0.0552	0.0529	0.0548	0.0775	0	0	-0.0025
B	0.8703	0.0809	0.123	0.1937	0.2411	0.1071	0.0617	0.0825	0.0691	0	-0.0001	-0.0023
C	0.4414	0.0677	0.0618	0.0851	3.4596	0.0515	0.0508	0.0527	0.0837	0.0002	0.0001	-0.002
D	0.2807	0.5582	0.0564	0.0918	3.4518	0.0781	0.1268	0.0631	3.4521	0	0	-0.0021
E	0.1744	0.0591	3.4892	0.0538	0.053	0.0507	0.0521	0.0705	0.0578	0	-0.0001	-0.0022
F	0.1145	0.0977	0.0516	3.3404	3.4857	0.0586	0.0695	0.063	0.0637	0	0	-0.0022
G	0.0589	0.0623	3.4769	3.468	0.0511	0.0643	0.0679	0.0557	0.0606	0.0001	0	-0.0022
H	0.0466	0.083	0.3824	0.0916	0.0513	0.0563	0.1319	0.0534	0.0516	-0.0001	0	-0.0027

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N1	N9	N17	N25			
B	Standard 50pg/ml	C2	C10	C18	C26	N2	N10	N18	N26			
C	Standard 25pg/ml	C3	C11	C19	C27	N3	N11	N19	N27			
D	Standard 12.5pg/ml	C4	C12	C20	C28	N4	N12	N20	N28			
E	Standard 6.25pg/ml	C5	C13	C21	C29	N5	N13	N21	N29			
F	Standard 3.125pg/ml	C6	C14	C22	C30	N6	N14	N22	N30			
G	STCM Control	C7	C15	C23	G1	N7	N15	N23	STCM Control			
H	Substrate Blank	C8	C16	C24	G2	N8	N16	N24	STCM Control			

Table 13. Setup of the p24 ELISA Assay for PT14 (1x10⁶ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight - positive well.

G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	2.2013	0.0589	0.0575	3.4682	0.0592	0.0682	0.0001	0.0001	0.0001	0.0001	0.0001	-0.0025
B	1.2022	2.1069	0.0605	0.0584	0.0574	0.0654	0.0001	0.0002	0.0001	0.0002	0.0001	-0.0021
C	0.6836	0.0576	0.6956	0.0554	0.0551	0.0693	0.0003	0.0003	0.0002	0.0002	0.0003	-0.002
D	0.3505	0.0579	0.0653	0.0618	0.0613	0.0591	0.0001	0.0002	0.0001	0.0002	0.0002	-0.0021
E	0.0561	0.0556	3.5055	0.0578	0.0594	0.0586	0.0001	0.0002	0.0001	0.0001	0.0001	-0.0023
F	0.0564	1.6904	0.06	0.0573	0.0564	0.0569	0.0001	0.0002	0.0001	0.0001	0.0001	-0.0022
G	0.0567	0.056	0.0558	0.0642	0.0588	0.0599	0.0001	0.0001	0.0001	0.0001	0.0002	-0.0022
H	0.0501	0.0569	0.057	0.1233	0.0613	0.1393	0.0001	0.0002	0.0001	0.0001	0.0001	-0.0026

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	N5	N13						
B	Standard 50pg/ml	C2	C10	C18	N6	N14						
C	Standard 25pg/ml	C3	C11	C19	N7	N15						
D	Standard 12.5pg/ml	C4	C12	C20	N8	N16						
E	G1	C5	C13	N1	N9	N17						
F	G2	C6	C14	N2	N10	N18						
G	STCM Control	C7	C15	N3	N11	N19						
H	Substrate Blank	C8	C16	N4	N12	N20						

Table 14. Setup of the p24 ELISA Assay for PT21 (1x10⁶ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight - positive well.

G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.5184	0.0508	0.0534	0.0543	0.0519	0.0497	0.0543	0.056	0.0577	-0.0001	-0.0001	-0.0026
B	0.7953	0.0521	0.0558	0.0519	0.0504	0.0522	0.0519	0.0521	0.0516	0.0001	0	-0.0023
C	0.4075	3.3516	0.049	0.7911	0.0531	0.0538	0.055	0.0525	0.0486	0.0002	0.0001	-0.002
D	0.2473	0.0477	3.4549	0.065	0.0503	0.0513	0.053	0.051	0.0512	0.0001	0.0001	-0.0021
E	0.1416	0.0513	0.0469	0.0483	0.7085	0.0571	0.0684	0.0534	0.048	0	-0.0001	-0.0023
F	0.0958	0.0481	0.0499	0.0499	0.0544	0.0495	0.0539	0.0484	0.05	0	0	-0.0021
G	0.0524	0.0471	0.0514	0.0476	0.0518	0.0529	0.0555	0.0633	0.0476	0	0.0001	-0.0021
H	0.0457	0.0486	0.0556	0.0499	0.049	0.0513	0.0556	0.0998	0.0496	0.0001	0	-0.0025

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N1	N9	N17	N25			
B	Standard 50pg/ml	C2	C10	C18	C26	N2	N10	N18	N26			
C	Standard 25pg/ml	C3	C11	C19	C27	N3	N11	N19	N27			
D	Standard 12.5pg/ml	C4	C12	C20	C28	N4	N12	N20	N28			
E	Standard 6.25pg/ml	C5	C13	C21	C29	N5	N13	N21	N29			
F	Standard 3.125pg/ml	C6	C14	C22	C30	N6	N14	N22	N30			
G	STCM Control	C7	C15	C23	G1	N7	N15	N23	STCM Control			
H	Substrate Blank	C8	C16	C24	G2	N8	N16	N24	STCM Control			

Table 15. Setup of the p24 ELISA Assay for PT40 (1x10⁶ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight - positive well.

G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.3895	0.0541	0.0501	0.0503	3.5731	0.051	0.05	0.0503	0.0493	0.0001	0.0002	-0.0025
B	0.8312	0.0539	0.0508	0.051	0.0518	0.0529	0.0858	0.0509	0.0499	0.0002	0.0001	-0.0023
C	0.4399	0.0525	0.0489	0.05	0.0495	0.0537	0.0488	0.0498	0.0502	0.0002	0.0002	-0.002
D	0.2608	0.0572	0.0487	0.0527	0.0497	0.0511	0.0513	0.0488	0.0484	0.0001	0.0001	-0.0021
E	0.1666	0.0628	0.0499	0.0507	0.0525	0.0525	0.0489	0.0512	0.049	0.0001	0.0001	-0.0023
F	0.1035	0.0539	3.518	0.049	0.0521	0.0497	0.0495	0.0527	0.0499	0.0001	0.0001	-0.0022
G	0.05	1.6187	0.0488	0.0493	0.0502	0.0496	0.0506	0.0491	0.0539	0.0002	0.0001	-0.0022
H	0.0456	3.6677	0.0485	0.0481	0.0505	0.056	0.0495	0.0538	0.0512	0.0001	0.0001	-0.0025

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N1	N9	N17	N25			
B	Standard 50pg/ml	C2	C10	C18	C26	N2	N10	N18	N26			
C	Standard 25pg/ml	C3	C11	C19	C27	N3	N11	N19	N27			
D	Standard 12.5pg/ml	C4	C12	C20	C28	N4	N12	N20	N28			
E	Standard 6.25pg/ml	C5	C13	C21	C29	N5	N13	N21	N29			
F	Standard 3.125pg/ml	C6	C14	C22	C30	N6	N14	N22	N30			
G	STCM Control	C7	C15	C23	G1	N7	N15	N23	STCM Control			
H	Substrate Blank	C8	C16	C24	G2	N8	N16	N24	STCM Control			

Table 16. Setup of the p24 ELISA Assay for PT40 (2x10⁵ cells/well) with spinoculation step and raw data

The overall setup for the p24 ELISA with raw data output. N – anti-CD3/CD28-beads-based assay. NS – anti-CD3/CD28-beads-based assay with spinoculation step.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.204	0.0528	0.0589	0.0522	0.0525	0.0529	0.052	0.0523	0.0542	0.0001	0.0001	-0.0025
B	0.5995	0.0544	0.0528	0.0534	0.0542	0.0552	0.0535	0.059	0.0529	0.0001	0.0001	-0.0023
C	0.3238	0.0511	0.0518	0.0524	0.0599	0.053	0.052	0.0526	0.0548	0.0003	0.0002	-0.002
D	0.1912	0.0511	0.0517	0.0554	0.0514	0.052	0.0552	0.0524	0.0533	0.0002	0.0001	-0.0021
E	0.1241	0.0518	0.053	0.0521	0.0513	0.0522	0.0535	0.0528	0.0622	0.0001	0	-0.0022
F	0.0833	0.0502	0.0502	0.0508	0.0507	0.0526	0.053	0.0538	0.0508	0.0001	0.0001	-0.0021
G	0.0527	0.0519	0.0563	0.0543	0.0633	0.0534	0.0547	0.0539	0.0578	0.0001	0.0001	-0.0022
H	0.051	0.0512	0.0521	0.0516	0.0528	0.0527	0.0531	0.0532	0.0537	0.0001	0.0001	-0.0025

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	N1	N9	N17	N25	NS1	NS9	NS17	NS25			
B	Standard 50pg/ml	N2	N10	N18	N26	NS2	NS10	NS18	NS26			
C	Standard 25pg/ml	N3	N11	N19	N27	NS3	NS11	NS19	NS27			
D	Standard 12.5pg/ml	N4	N12	N20	N28	NS4	NS12	NS20	NS28			
E	Standard 6.25pg/ml	N5	N13	N21	N29	NS4	NS13	NS21	NS29			
F	Standard 3.125pg/ml	N6	N14	N22	N30	NS6	NS14	NS22	NS30			
G	STCM Control	N7	N15	N23	STCM Control	NS7	NS15	NS23	STCM Control			
H	Substrate Blank	N8	N16	N24	STCM Control	NS8	NS16	NS24	STCM Control			

Table 17. Setup of the p24 ELISA Assay for PT21 (2x10⁵ cells/well) with spinoculation step and raw data, well 1 through 30

The overall setup for the p24 ELISA with raw data output. N – anti-CD3/CD28-beads-based assay. NS – anti-CD3/CD28-beads-based assay with spinoculation step.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.2647	0.0531	0.0549	0.0683	0.0535	0.0573	0.0542	0.0537	0.0565	0.0001	0	-0.0029
B	0.716	0.0583	0.0596	0.0545	0.0688	0.064	0.0543	0.0617	0.0588	0.0001	0.0001	-0.0025
C	0.3925	0.0553	0.0513	0.0517	0.0526	0.0553	0.0525	0.0536	0.0569	0.0002	0.0001	-0.0024
D	0.2155	0.0555	0.0544	0.0523	0.0514	0.0524	0.0558	0.0592	0.0517	0.0002	0	-0.0025
E	0.1405	0.0518	0.0552	0.0513	0.0544	0.0521	0.0523	0.0524	0.0521	0.0001	0	-0.0027
F	0.0891	0.0541	0.0511	0.0509	0.0543	0.052	0.0514	0.0516	0.0737	0.0001	0.0001	-0.0025
G	0.0522	0.0532	0.0509	0.0512	0.053	0.0549	0.0546	0.0526	0.0546	0.0002	0.0001	-0.0025
H	0.0505	0.0513	0.0541	0.0518	0.0526	0.0597	0.0631	0.0531	0.0517	0	0.0001	-0.0028

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	N1	N9	N17	N25	NS1	NS9	NS17	NS25			
B	Standard 50pg/ml	N2	N10	N18	N26	NS2	NS10	NS18	NS26			
C	Standard 25pg/ml	N3	N11	N19	N27	NS3	NS11	NS19	NS27			
D	Standard 12.5pg/ml	N4	N12	N20	N28	NS4	NS12	NS20	NS28			
E	Standard 6.25pg/ml	N5	N13	N21	N29	NS4	NS13	NS21	NS29			
F	Standard 3.125pg/ml	N6	N14	N22	N30	NS6	NS14	NS22	NS30			
G	STCM Control	N7	N15	N23	STCM Control	NS7	NS15	NS23	STCM Control			
H	Substrate Blank	N8	N16	N24	STCM Control	NS8	NS16	NS24	STCM Control			

Table 18. Setup of the p24 ELISA Assay for PT21 (2x10⁵ cells/well) with spinoculation step and raw data, well 31 through 45

The overall setup for the p24 ELISA with raw data output. N – anti-CD3/CD28-beads-based assay. NS – anti-CD3/CD28-beads-based assay with spinoculation step.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.3869	0.0579	0.0545	0.054	0.0542	0.0001	0.0001	0	0.0001	0.0001	0.0001	-0.0024
B	0.7276	0.0645	0.0677	0.0688	0.0548	0.0001	0.0001	0.0001	0.0001	0.0002	0.0001	-0.0021
C	0.4211	0.055	0.0614	0.0536	0.0525	0.0003	0.0001	0.0002	0.0002	0.0003	0.0002	-0.0018
D	0.2484	0.0549	0.0558	0.0616	0.0537	0.0002	0.0001	0.0001	0	0.0002	0.0001	-0.0019
E	0.2657	0.0557	0.053	0.0537	0.0536	0.0001	0	0.0001	0	0.0002	0	-0.0021
F	0.1085	0.0552	0.0652	0.0543	0.068	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	-0.0019
G	0.0537	0.0554	0.0543	0.0552	0.0672	0.0002	0.0001	0.0002	0.0001	0.0001	0.0002	-0.0019
H	0.0472	0.0612	0.0563	0.0574	0.0566	0	0.0001	0.0002	0.0001	0.0001	0.0001	-0.0025

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	N1	N9	NS1	NS9							
B	Standard 50pg/ml	N2	N10	NS2	NS10							
C	Standard 25pg/ml	N3	N11	NS3	NS11							
D	Standard 12.5pg/ml	N4	N12	NS4	NS12							
E	Standard 6.25pg/ml	N5	N13	NS4	NS13							
F	Standard 3.125pg/ml	N6	N14	NS6	NS14							
G	STCM Control	N7	N15	NS7	NS15							
H	Substrate Blank	N8	STCM Control	NS8	STCM Control							

Table 19. Setup of the p24 ELISA Assay for ES31 (1x10⁶ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.7603	0.055	0.0545	0.0565	0.0546	-0.0003	-0.0003	-0.0003	-0.0003	-0.0005	-0.0005	-0.0031
B	0.4553	0.0566	0.0534	0.0545	0.0558	-0.0003	-0.0003	-0.0002	-0.0003	-0.0004	-0.0005	-0.0028
C	0.2587	0.055	0.0536	0.0539	0.0594	0	-0.0002	-0.0002	-0.0002	-0.0003	-0.0005	-0.0027
D	0.1677	0.0542	0.0565	0.0546	0.0535	0	-0.0002	-0.0004	-0.0003	-0.0005	-0.0005	-0.0028
E	0.121	0.0548	0.0578	0.0552	0.0537	0	-0.0006	-0.0005	-0.0005	-0.0004	-0.0006	-0.0029
F	0.0558	0.0541	0.0543	0.0548	0.056	-0.0001	-0.0005	-0.0005	-0.0005	-0.0006	-0.0005	-0.0028
G	0.0552	0.0539	0.0592	0.0547	0.0547	0	-0.0005	-0.0005	-0.0005	-0.0004	-0.0005	-0.0028
H	0.0519	0.0567	0.0569	0.0541	0.054	0	-0.0005	-0.0005	-0.0005	-0.0005	-0.0004	-0.0031

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	N8	N16						
B	Standard 50pg/ml	C2	C10	N1	N9	N17						
C	Standard 25pg/ml	C3	C11	N2	N10	N18						
D	Standard 12.5pg/ml	C4	C12	N3	N11	G1						
E	Standard 6.25pg/ml	C5	C13	N4	N12	G2						
F	Standard 3.125pg/ml	C6	C14	N5	N13	STCM Control						
G	STCM Control	C7	C15	N6	N14	STCM Control						
H	Substrate Blank	C8	C16	N7	N15	STCM Control						

Table 20. Setup of the p24 ELISA Assay for ES23 (1x10⁶ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.7371	0.053	0.0515	0.0515	0.0507	0.0523	0.0001	0.0001	0	0.0001	0.0001	-0.0025
B	0.391	0.0539	0.0537	0.0521	0.0512	0.0518	0.0001	0.0002	0.0001	0.0001	0.0001	-0.0022
C	0.2272	0.0535	0.0533	0.0518	0.057	0.0546	0.0002	0.0002	0.0002	0.0003	0.0001	-0.0019
D	0.1357	0.0555	0.0582	0.0553	0.0538	0.055	0.0001	0.0001	0.0001	0.0002	0.0001	-0.0021
E	0.116	0.0542	0.0547	0.0525	0.0542	0.0674	0	0.0001	0	0.0001	0	-0.0022
F	0.0742	0.0528	0.0521	0.055	0.0519	0.0522	0.0001	0.0001	0.0001	0.0001	0.0001	-0.0021
G	0.053	0.0543	0.0582	0.0529	0.0534	0.0555	0.0001	0.0001	0.0001	0.0001	0.0001	-0.0021
H	0.0473	0.06	0.0599	0.0545	0.0527	0.0551	0.0001	0	0.0001	0.0001	0.0001	-0.0025

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	N8	N16						
B	Standard 50pg/ml	C2	C10	N1	N9	N17						
C	Standard 25pg/ml	C3	C11	N2	N10	N18						
D	Standard 12.5pg/ml	C4	C12	N3	N11	G1						
E	Standard 6.25pg/ml	C5	C13	N4	N12	G2						
F	Standard 3.125pg/ml	C6	C14	N5	N13	STCM Control						
G	STCM Control	C7	C15	N6	N14	STCM Control						
H	Substrate Blank	C8	C16	N7	N15	STCM Control						

Table 21. Setup of the p24 ELISA Assay for ES46 (1x10⁶ cells/well) and raw data

The overall setup for the p24 ELISA with raw data output. C – standard PHA/feeders assay. N – anti-CD3/CD28-beads-based assay. Yellow highlight - positive well.

G – controls.

RAW DATA												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.976	0.0542	0.0537	0.0529	0.0578	0.0542	0.055	0.0527	0.0531	0	0	-0.0026
B	0.5329	0.0525	0.0578	0.0528	0.0522	0.0519	0.0543	0.0529	0.0536	0.0001	0	-0.0023
C	0.3236	0.0578	0.0518	0.0525	0.0593	0.0624	0.0566	0.053	0.0525	0.0002	0.0001	-0.0019
D	0.197	0.0569	0.0554	0.0558	0.0562	0.0546	0.0537	0.0544	0.0538	0.0001	0	-0.0021
E	0.1223	0.0613	0.054	0.0553	0.0536	0.0535	0.0539	0.0594	0.0541	0.0001	0	-0.0022
F	0.0902	0.0581	0.054	0.0536	0.0548	0.0532	0.0536	0.0532	0.0538	0	0.0001	-0.0022
G	0.0525	0.0534	0.0531	0.054	0.054	0.053	0.0561	0.0539	0.053	0.0001	0	-0.0022
H	0.0558	3.6888	0.0541	0.0536	0.0528	0.0538	0.0542	0.0536	0.0531	0.0001	0.0001	-0.0025

P24 PLATE SETUP												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 100pg/ml	C1	C9	C17	C25	N1	N9	N17	N25			
B	Standard 50pg/ml	C2	C10	C18	C26	N2	N10	N18	N26			
C	Standard 25pg/ml	C3	C11	C19	C27	N3	N11	N19	N27			
D	Standard 12.5pg/ml	C4	C12	C20	C28	N4	N12	N20	N28			
E	Standard 6.25pg/ml	C5	C13	C21	C29	N5	N13	N21	N29			
F	Standard 3.125pg/ml	C6	C14	C22	C30	N6	N14	N22	N30			
G	STCM Control	C7	C15	C23	G1	N7	N15	N23	STCM Control			
H	Substrate Blank	C8	C16	C24	G2	N8	N16	N24	STCM Control			

CHAPTER 4 – DISCUSSION AND FUTURE DIRECTIONS

We demonstrated that the anti-CD3/CD28-bead-based assay is capable of quantifying the HIV-1 latent reservoir in resting CD4⁺ T cells with a similar sensitivity compared to that achieved by the conventional PHA/feeders QVOA. While the former induces a more rapid stimulation of target cells than the PHA/feeders assay, the latter was more efficient at amplifying virus when replicates of 2×10^5 were tested. Of note, the 3 patients from whom virus was selectively not amplified with the bead-based assay had relatively low frequencies of latently infected cells in the PHA/feeders assay. Just 1 out of 25 wells was positive for PT14 and PT21 and 3 out of 30 wells were positive for PT8. When higher numbers of cells were tested, virus was amplified from all 4 patients using the PHA/feeders assay and 2 patients with the bead-based assay. Again, the percentage of positive wells in PHA/feeders assay in these 2 patients (PT21 and PT40) was relatively low (4 out of 30 wells). Thus, while there was not a significant difference in the sensitivity between the 2 assays, the PHA/feeders QVOA appeared to be slightly more effective at viral amplification in some patients. Interestingly enough, one of these patients was a long-term non-progressor, which could explain the inability of the anti-CD3/CD28 assay to detect virus. As shown in the Table through Table 21, the microbeads-based assay was not able to detect any amount of virus either. However, given the fact that the second patient in the anti-CD3/CD28 spinoculation attempt was not a controller, there seems to be a different explanation for the lack of viral amplification. First, it is possible that stimulation with microbeads does not achieve a similar level of global T cell activation compared to the PHA. Secondly, the addition of allogeneic feeders serves as a secondary

stimulus for activation, possibly due to the presence of macrophages and dendritic cells. However, data in Figure 5 seems to show faster activation in response to the anti-CD3/CD28 beads and not the PHA.

The advantage of the PHA/feeders assay in being more effective at viral amplification in some patients must be balanced against the fact that the need for feeders and a PHA washout step makes it more labor intensive and costly than the bead-based assay. An additional measure for simplifying the bead-based assay would be to use a cell line such as MOLT-4/CCR5 instead of lymphoblasts from healthy donors for amplifying virus as previously described for the PHA/feeders assay (Laird et al., 2013). The bead-based assay would also be easier to use for the repeated stimulation of CD4⁺ T cells that is needed to trigger replication of the non-induced viral clones that are refractory to a single round of T cell activation (Ho et al., 2013).

The CD3/CD28 assay presented here represents a less expensive and less labor intensive approach while maintaining the sensitivity similar to that of the conventional QVOA. As such, we strongly believe this assay will be valuable in evaluating strategies targeting latency reversal agents in large-scale clinical trials.

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APPENDIX

MATERIALS

1. Wash Media

To Make Stock

3L 10x PBS pH 7.2-7.4 – Invitrogen 70011044

6x 500mL bottles

600mL New Born Calf Serum – Invitrogen 26010074

1x 500mL bottles

1x 100mL from 2nd bottle

300mL 10% w/v dextrose – Fisher 118058

30g/300mL

Mix 250mL Deionized Water and 30g – stir on plate

Use graduated cylinder to adjust volume to 300mL

300mL Pen-Strep – 15140-122

3x 100mL bottle

360mL Hepes 1M pH 7.2 – 15630-080

3x 100mL bottle

1x 60mL from 4th bottle

Using 6L flask

Add 100 NBCS and 60mL Hepes

Add remaining ingredients (add remaining NBCS last)

Aliquot 456mL/500mL autoclaved bottle – do not filter

6x store in -20C

2x store in 4C

2x dilute to working concentration

To Make Working Solution

In 6L flask

Add 1 stock (456mL)

Add MilliQ water to 3L

Filter into 1L bottles using bottle top filter

Store at 4C (cold room)

2. Heparin

100x (Working Solution)

Order from Sigma-Aldrich, Cat #3149-100ku

1. Fill 2 50mL conical tubes with 1x PBS (pH 7.2)
2. Add 10mL from conical to the Heparin container
3. Mix, remove, put into 100mL beaker
4. Repeat steps 2 and 3 until all 100mL has been used
5. Put 50mL solution back into each 50mL conical tube
6. Steriflip both
7. Aliquot 1.8mL into 2mL flat bottom cryovials (optional)
8. Store at 4C

3. B-Cell Media (BCM)

500mL RPMI (Invitrogen 61870036)

5.5mL Penn/Strep (Invitrogen 15140122)

55mL Heat Inactivated (HI) FBS (Gemini 100-106)

4. Super T-Cell Media (STCM)

500mL RPMI (Invitrogen 61870036)

5.5mL Penn/Strep (Invitrogen 15140122)

55mL Heat Inactivated (HI) FBS (Gemini 100-106)

T-Cell Growth Factor (~5-10mL) *for further preparation instructions, refer to (Siliciano & Siliciano, 2005)

240 uL IL-2 (Final Concentration 100U/mL)

-spin down aliquot before using

IL-2 is called Proleukin (Novartis), order from Hopkins Pharmacy

5. Phytohaemagglutinin (PHA)

150 ug/ml

Manufacturer Remel Inc.

Order from Fisher, Cat # R30852801

Work in the hood to make the following:

PHA comes as 2mg/bottle

- Add 5 ml wash media to the bottle
- Transfer to 50 ml conical tube (Be sure to rinse around the lid for powder residue)
- Add 8.3 ml wash media to the bottle (wash)
- Transfer to the same 50 ml conical tube (FV = 13.3 ml)
- Use steriflip to filter
- Aliquot 0.5 ml into conical 1ml cryovials
- Store in -80C
- After thawing, PHA is only good for 2 weeks at 4C

METHODS

1. Counting Cells Using Hemocytometer

Materials:

1. Hemocytometer (any standard with four 16 corner squares is sufficient)
2. Trypan blue viability dye (Gibco 15250061)
3. 96-well round bottom plate for mixing cells and viability dye

Methods

1. Prepare cell-trypan blue dilution (50 ul : 50 ul = 2-fold dilution; 10 ul : 90 ul = 10-fold dilution) in a 96-well plate
2. Place the mixture in the hemocytometer
3. Calculate the average number of cells from two sets of the 16 corner squares
4. Multiply by 10,000 (10^4)
5. Multiply by the appropriate dilution factor

2. Lymphocyte Isolation by Ficoll Gradient

Materials

1. Ficoll-Paque Plus (GE Healthcare, 71-7167-00 AG)
2. Appropriate tubes and pipettes
3. Wash media (WM)

Methods

1. In 50 ml conical tube place 15 ml ficoll
2. Slowly overlay 30 ml of blood on top of the ficoll layer being careful not to disturb the ficoll layer (use slow setting on the pipette)
3. Spin at 1300 RPM for 35 mins at room temperature with NO BRAKE
4. During spin, prepare appropriate number of 50 ml conical tubes with wash media for the next step
5. You will then see a gradient with plasma on top, lymphocytes in a white fluffy layer, ficoll, then RBCs at the bottom of the tube
6. Aspirate the top layer (or keep the plasma if experiments asks for it) to about 5 ml above the lymphocyte layer
7. Using a 10 ml pipette carefully remove the white fluffy layer, being careful not to take any RBCs
8. Place lymphocytes in the conical tubes from step 4
9. Spin at 1400 RPM for 12 mins at 4C
10. If too many RBC, can ACK treat (consult the experimental directions before doing it)

11. If more than one tube in the previous step, pool the pellets together in one tube
with appropriate amount of wash media
12. Spin at 1400 RPM for 12 mins at 4C
13. Resuspend in appropriate media and count

3. Isolation and Purification of CD4⁺ T cells

Materials:

1. Human CD4⁺ T Cell Isolation Kit, Miltenyi Biotec. 130-096-533
2. LS columns (Miltenyi Biotec, 130-042-401)
3. Magnetic Field, Miletnyi 130-042-303
4. Wash Media (WM)
5. Appropriate media for downstream applications, conical tubes and pipettes

Methods:

1. After washing PBMCs, perform steps below (per 10⁷ cells)
2. Add:
 - 40 ul of WM
 - 10 ul CD4 cocktail beads
3. Mix and incubate for 5 min at 4C
4. Add:
 - 30 ul of WM
 - 20 ul of anti-biotin microbeads
5. Mix and incubate for 10 min at 4C
6. Place appropriate number of LS columns in the magnetic field and put a 50ml conical tube under the column
7. Wash with 3 ml cold wash media
8. Add the cells (max 150x10⁶ cells per LS column)
9. Do two more washes of the LS column with cold WM
10. Count the CD4⁺ T cells.

11. Spin cells at 1200RPM for 10 min at 4C
12. Resuspend cells in appropriate media for downstream applications

4. Isolation and Purification of resting CD4⁺ T cells

Materials:

1. Anti-HLA-DR Microbeads, Miltenyi Biotec
2. CD25 Microbeads, Miltenyi Biotec
3. CD69 Microbead Kit II, Miltenyi Biotec
4. LS columns (Miltenyi Biotec, 130-042-401)
5. Magnetic Field, Miltenyi 130-042-303
6. Wash Media (WM)
7. Appropriate media for downstream applications, conical tubes and pipettes

Methods:

1. For depletion of activated CD4⁺ T cells, perform steps below (per 10⁷ cells)
2. Add:
 - 40 ul of WM
 - 10 ul of CD69 beads
3. Mix and incubate for 15 min at 4C
4. Add:
 - 30 ul of WM
 - 20 ul of anti-CD69 microbeads
 - 10 ul of CD25 beads
 - 10 ul of HLA-DR beads
5. Mix and incubate for 15 mins
6. Add 30 ml of cold WM and spin for 10 min at 1200 RPM
7. Resuspend the resting CD4⁺ T cells in 500 ul of cold WM

8. Place appropriate number of LS columns in the magnetic field and put a 50ml conical tube under the column
9. Wash with 3 ml cold wash media
10. Add the cells from step 7 (max 150×10^6 of initial cells from step 1 per LS column)
11. Do two more washed of the LS columns with cold WM
12. Count the resting CD4⁺ T cells
13. Spin cells at 1200 RPM for 10 min at 4C
14. Resuspend purified resting CD4⁺ T cells in appropriate media for downstream applications

5. Preparation of Irradiated PBMC (Feeders)

Materials

1. Ficoll-Paque Plus (GE Healthcare, 71-7167-00 AG)
2. Appropriate tubes and pipettes
3. Wash media (WM)
4. PHA
5. STCM
6. Bucket with ice

Methods:

1. Collect healthy donor blood (120-180 ml depending on the experiment) and irradiate the blood to inactivate the cells (10 fold higher number of irradiated PBMCs than purified resting CD4⁺ T cells are needed) γ - irradiation 14 min with 5000R in a Cesium source irradiator
2. Ficoll separate PBMC from whole blood and wash according to Methods 2.
3. Resuspend PBMCs in STCM at appropriate concentration
4. PHA is added to the cell suspension at 1 ug/ml (final concentration in the co-culture = 0.5 ug/ml)
5. Tube is immediately placed on ice to prevent macrophages from adhering to the tub

6. Removal of PHA and Addition of Blasts to Culture Assay

Materials:

1. Appropriate conical tubes and pipettes
2. STCM

Methods:

1. Carefully remove media without disturbing the cells:
 - 6-well plate: 6 ml removed
 - 24-well plate: 1.5. ml removed
2. Replace with the same quantity of STCM (fill slowly)
3. Let cells settle for at least 3 hours.
4. Isolate CD4⁺ lymphoblasts from the earlier preparation
5. Add the appropriate number of CD4⁺ T cell lymphoblasts
 - 6-well plate: 4x10⁶ cells are added in 6 ml STCM. This amount is optimal, can do with half the amount (in the same volume of STCM) with limited numbers
 - 24-well plate: 1x10⁶ cells are added in 1.5 ml STCM. This amount is optimal, can do with half the amount (in the same volume of STCM) with limited numbers

7. Production of CD4⁺ Lymphoblasts (Blasts)

Materials:

1. Ficoll-Paque Plus (GE Healthcare, 71-7167-00 AG)
2. Appropriate tubes and pipettes
3. Wash media (WM)
4. STCM
5. PHA

Methods:

1. Ficoll healthy donor PBMC from whole blood (60-180 ml)
2. After the pellet has been washed adequate times, resuspend it in 30 ml STCM
3. Add 100 ul PHA
4. Transfer cells to a 75 ml flask
5. Incubate upright for 2-3 days at 37C under 5% CO₂

8. Isolation and Purification of CD4⁺ T cells lymphoblasts

Materials

1. Human CD4⁺ T Cell Isolation Kit, Miltenyi Biotec. 130-096-533
2. LS columns (Miltenyi Biotec, 130-042-401)
3. Magnetic Field, Miletnyi 130-042-303
4. Appropriate tubes and pipettes
5. Wash media (WM)
6. STCM

Methods:

1. Add the PBMC in PHA from earlier step (30 ml) into a 50 ml conical tube and wash the flask with 10 ml cold WM
2. Count cells
3. Centrifuge cell suspension at 1400 RPM for 12 min at 4C
4. After washing PMBC, isolate CD4⁺ T cells as described in Method 3
7. Add appropriate amount of STCM for the final concentrations as determined by the amount of wells plated

9. p24 ELISA

Materials:

1. P24 ELISA kit
2. 24-well plate = 3.4 ml/well (48-well plate = 1.6ml/well)
3. Wash media (WM)

Things to do before starting:

1. Reagent preparation
 - a. Equilibrate all reagents to room temp (15-30C) before use
2. Plate wash concentrate (WC)
 - a. Make 1X from stock = 1 part WC + 19 H₂O
 - i. ~1000ml of WC is used per plate assayed

Methods:

1. Prepare standard
 - a. Label wells of a 24-well plate A-G
 - b. Add 980 ul WM to A
 - c. Add 975 ul WM to B
 - d. Add 500ul WM to C-G
 - e. Add 20ul p24 standard to A
 - f. Mix with p1000 by pipetting up and down to the max
 - g. Transfer 25ul of A to B
 - h. Mix with p1000 by pipetting up and down to the max
 - i. Serial dilute from B-F with 500ul
2. Prepare sample in a 96-well plate

- a. Perform dilutions if necessary (concentrated virus normally $1:10^5$ – $1:10^8$ range work, use a necessary volume and corresponding amounts of the virus)
 - i. $1:10^2 \Rightarrow 297\text{ul WM} + 3\text{ul Virus stock} = A$
 - ii. $1:10^3 \Rightarrow 270\text{ul WM} + 30\text{ ul A} = B$
 - iii. $1:10^4 \Rightarrow 270\text{ul WM} + 30\text{ ul B} = C$
 - iv. $1:10^5 \Rightarrow 270\text{ul WM} + 30\text{ ul C} = D$
 - v. $1:10^6 \Rightarrow 270\text{ul WM} + 30\text{ ul D} = E$
 - vi. $1:10^7 \Rightarrow 270\text{ul WM} + 30\text{ ul E} = F$
 - vii. $1:10^8 \Rightarrow 270\text{ul WM} + 30\text{ ul F} = G$
3. Collect necessary strips of mAb-coated wells (need a standard for each plate)
4. Add 20ul Triton to each well
5. Add 180ul of sample (including standard) to each well
6. Incubate at 37C for 1-2 hrs (can do overnight if necessary – in this case store in the fridge at 4C)
7. Wash – plate washer ideal (using p24 wash) – blot well
8. Add 100ul detector Ab to each well
9. Incubate at 37C for >45 mins
10. Wash – blot well
11. Add 100ul streptavidin mixture
 - a. 10ul concentrate + 1ml diluent
 - b. Make 1 ml per strip – adjust accordingly
12. Incubate at room temp > 25mins

13. Wash – blot well
14. Add 100ul substrate
 - a. 11ml diluent + 1 tablet
 - b. Make 2 if necessary
 - c. PROTECT FROM LIGHT
15. Incubate at room temp for > 25mins
 - a. PROTECT FROM LIGHT
16. Add 100ul stop solution
 - a. Read within 15 mins of stopping the reaction
17. Read plate
 - a. 490 or 492
10. Shake

10. Staining

Materials:

1. FACT tubes (5 ml)
2. DynaMag – 5 Magnet (ThermoFisher Scientific, 12303D)
3. PBS
4. Appropriate antibodies
5. Cytofix/Cytoperm (BD, 554714)

Methods:

Removal of the microbeads:

1. For each sample, first remove the dynabeads:
2. Transfer each well into its own 5 mL FACS tube.
3. Spin down for 5 minutes at 1400 rpm at 4°C.
4. Remove all but 200 uL of media and then resuspend the pellet.
5. Place the FACS tube in the magnetic tube rack and wait for one minute to allow for the magnetic beads to attach to the side
6. Transfer the cell suspension (sans dynabeads) to another 5 mL FACS tube.
7. Repeat for each sample.

Staining Procedure (for microbead-treated cells, in our case $<1 \times 10^6$ cells total)

1. Spin down tubes at 1400 rpm for 3 minutes at 4°C.
2. Wash with PBS and spin down again.
3. In the meantime, make up the master stain in PBS:
 - a. 50 μ L of PBS for each well.
 - b. 1 μ L of each stain per well (ex. if three antibodies, add 1 μ L of each stain).

- c. For this activation panel, add:
 - i. CD3-PacBlue, CD8-APC•H7, CD4-PE
 - ii. HLA-A2-PerCP•Cy5.5
 - iii. CD25-FITC, HLA-DR-APC, CD69-BV605
4. Remove supernatant from tubes and add 50 μ L master stain to each well.
5. Incubate for 25 minutes at 4°C on ice.
6. Add 1mL of PBS to each tube and spin down.
7. Remove supernatant and wash an additional two times with 1mL each.
8. Resuspend in 100 μ L PBS and run on the FACS Canto.
 - a. Can fix cells (resuspend in 200 μ L of fixative – Cytofix/Cytoperm) at this point and run FACS later (need to wash off before running the samples)

For PHA and feeders stimulated wells (in our case $>1 \times 10^6$ cells)

1. Spin down cells in a FACS tube
2. Make up master stain in PBS
 - a. 50 μ L of PBS for each well.
 - b. 5 μ L of each stain per well (ex. if three antibodies, add 5 μ L of each stain).
- c. For this activation panel, add:
 - i. CD3-PacBlue, CD8-APC•H7, CD4-PE
 - ii. HLA-A2-PerCP•Cy5.5
 - iii. CD25-FITC, HLA-DR-APC, CD69-BV605
3. Remove supernatant from tubes and add 80 μ L master stain to each well.
4. Incubate for 25 minutes on ice.
5. Add 1mL of PBS to each well and spin down.

6. Remove supernatant and wash an additional two times with 1mL each.
7. Resuspend in 100 μ L PBS and run on the FACS Canto.
 - a. Can fix cells (resuspend in 200 μ L of fixative – Cytofix/Cytoperm) at this point and run FACS later (need to wash off before running the samples)

11. FACS

Methods

1. Check fluid levels
2. Turn on the machine
3. Perform fluidics startup with HTS attached
4. Set up experiment
 - i. Keywords
 - ii. Color labels
5. Run sample
 - i. Flow rate should be $< 10,000$ events per second
 - ii. If cells have been sitting, resuspend
 - iii. When selecting volume for HTS, always leave 25% of sample
6. If last user for the day, perform fluidics shutdown with HTS attached
7. After shutdown complete, turn off machine
8. Export FCS files
9. Analyze data on FlowJo

CURRICULUM VITAE

YURY V. KUZMICHEV

Johns Hopkins University Bloomberg School of Public Health +1 (620) 704-1416
Department of Molecular Microbiology and Immunology ykuzmic1@JHU.edu
615 N. Wolfe St. Room E5132 yuryvk@GMAIL.com
Baltimore, MD 21205 <http://yuryvkuzmichev.com>

EDUCATION

Johns Hopkins Bloomberg School of Public Health
ScM in Molecular Microbiology and Immunology

Thesis title: “A Novel Anti-CD3/CD28 Bead-Based PHA/Feeder-Cell-Free Human Immunodeficiency Virus-1 Outgrowth Assay” *Baltimore, MD*
2016

Advisor: Dr. Joel N. Blankson

Johns Hopkins Bloomberg School of Public Health
Vaccine Science and Policy Certificate

Baltimore, MD
2016

Pittsburg State University
BS cum laude in Chemistry (ACS Certified) and Biology

Pittsburg, KS
2009

RESEARCH EXPERIENCE

Johns Hopkins Bloomberg School of Public Health,
Department of Molecular Microbiology and Immunology

Baltimore, MD

Johns Hopkins University School of Medicine, Division of
Infectious Diseases, Department of Medicine

Nov. 2014 – May 2016

Masters Student in the laboratory of Dr. Joel N. Blankson

- Development of an improved version of the conventional Quantitative Viral Outgrowth Assay (QVOA) by means of alternative activation of CD4⁺ T cells and consequent elimination of the feeder cells
- Cloning and sequence characterization of HIV NL4-3 constructs with Gag sequences derived from HIV-infected individuals with undetectable viral loads currently on or without therapy
- Analysis of the CTL response to Gag clones derived from HLA-B-57+ HIV-infected individuals using suppression assays followed by appropriate flow cytometric analysis

Harvard Medical School, New England Primate Research Center, Division of Immunology

Southborough, MA

Research Assistant in the laboratory of Dr. R. Paul Johnson

Sept. 2009 – Jan. 2012

- Identification and characterization of novel molecular interactions in HIV models in primates with focus on NKG2D receptor and its corresponding ligands
- Organization and implementation of novel protocols for the optimization of custom TaqMan probes for use in real-time PCR assays on various platforms (Applied Biosystems, Fluidigm)
- Refinement of the analysis of high-throughput real-time PCR raw data (Fluidigm) with custom written VBA
- Development of novel bioinformatics protocols with incorporation of Geneious software with implementation of cross-sharing capabilities for sequence analysis using a localized SQL server (Streamlined protocols for the development of custom TaqMan probes for use in real-time PCR assays)

Harvard Medical School, New England Primate Research Center, Division of Immunology

Southborough, MA

Summer Research Internship in the laboratory of Dr. R. Paul Johnson

May – Aug. 2008

- Performed ficoll gradient isolation of lymphocytes from the whole blood following standard protocols
- Assisted in lymphocyte collection and enrichment for NK cells by negative selection magnetic bead separation
- Identified potential regions for sequence-specific primers (SSPs) for killer immunoglobulin-like receptors (KIRs) by performing multiple mRNA sequence analysis

Vanderbilt University, Department of Chemistry

Nashville, TN

Summer Research Internship in the laboratory of Dr. Michael P. Stone

May – Aug. 2007

- Obtained 1-D and 2-D NMR spectra of double-stranded B-DNA duplex on Bruker Spectrometers
- Analysed and assigned corresponding NMR peaks (NOESY, COSY) for downstream structural analysis
- Refined the final structure of the duplex oligonucleotide, generated in Insight II, by measuring NOESY Cross Peak Volumes
- Calculated interproton distances by Relaxation Matrix Analysis (MARDIGRAS)
- Derived a semi-final 3D structure of the duplex using Restrained Molecular Dynamics in the AMBER force field

**Moscow Research Institute MONIKI, Department of
Thoracic Surgery**

Moscow, Russia

*Summer Research Internship in under Dr. Vladimir
Aleksandrovich*

May – Aug. 2006

- Maintained patient history and improved the efficiency of database entries by rewriting the SQL code
- Conducted statistical analysis on the outcomes of treatment of patients suffering from complex regional pain syndrome

PUBLICATIONS

Articles

1. “Characterization of CD8+ T Cell Differentiation Following SIV Δ nef Vaccination by Transcription Factor Expression Profiling”. James M. Billingsley, Premeela A. Rajakumar, Michelle A. Connole, Nadine C. Salisch, **Yury V. Kuzmichev**, Henoch S. Hong, R. Keith Reeves, Hyung-joo Kang, Wenjun Li, Ashley T. Haase, R. Paul Johnson. PLoS Pathog, 11(3), e1004740. doi: 10.1371/journal.ppat.1004740 PubMed Central PMCID: 25768938
2. “Gut inflammation and indoleamine deoxygenase inhibit IL-17 production and promote cytotoxic potential in NKp44+ mucosal NK cells during SIV infection.” Reeves RK, Rajakumar PA, Evans TI, Connole M, Gillis J, Wong FE, **Kuzmichev YV**, Carville A, Johnson RP. Blood. 2011;118(12):3321-30. Epub 2011/07/28. doi: 10.1182/blood-2011-04-347260. PubMed PMID: 21791421; PubMed Central PMCID: PMC3179400.

Conference Proceedings

1. “Maturation of protective immunity induced by SIV Δ nef correlates with differential expression of transcription factors in SIV-specific CD8+ T cells.” Billingsley J, Rajakumar P, Salisch N, **Kuzmichev Y**, Hong H, Connole M, RK Reeves, H Kang, W Li, RP Johnson. Retrovirology. 2012;9(Suppl 2):P33. PubMed PMID: doi:10.1186/1742-4690-9-S2-P33.
2. “Transcription factor expression profiling to characterize differentiation of antigen-specific CD8 T cells induced by a live attenuated viral vaccine.” Billingsley J, Rajakumar P, Salisch N, **Kuzmichev Y**, Hong H, Connole M, RK Reeves, R. Johnson. The Journal of Immunology, May 2012, 188, 113.18
3. “Gut Inflammation and Indoleamine Deoxygenase Inhibit I-17 Production and Induce Cytotoxicity in ROR gamma t+ NKp44+ Mucosal NK Cells During SIV Infection.” R. Reeves, P. Rajakumar, T. I. Evans, M. Connole, J. Gillis, **Y. Kuzmichev**, R. Johnson. Conference Paper in AIDS Research and Human Retroviruses. Oct. 2011

4. "Differential Expression of Transcription Factors in SIV-Specific CD8+ T cells at Week 5 and Week 20 After Vaccination with SIV239 Delta nef." J. M. Billingsley, P. A. Rajakumar, N. C. Salisch, **Y. V. Kuzmichev**, M. A. Connole, R. K. Reeves, R. P. Johnson. Conference Paper in AIDS Research and Human Retroviruses. Sep. 2011
5. "Evaluation of coumarin derivatives as anti-fungal agents against soil-borne fungal pathogens." Brooker NL, **Kuzmichev Y**, Laas J, Pavlis R. Communications in agricultural and applied biological sciences. 2007;72(4):785-93. PubMed PMID: 18396811.

PRESENTATIONS

Conference/Poster Presentations

1. "Quantification of HIV-1 Latent Reservoir Using Physiological Activation of CD4+ T Cells." **Yury V. Kuzmichev**, Christopher W. Pohlmeier, Victoria E. K. Walker-Sperling, and Joel N. Blankson. Poster session at the 32nd Clinical Virology Symposium, meeting of the American Society of Microbiology, Daytona Beach, FL, May 19-22, 2016. Poster session at the 2016 Department of Medicine Research Retreat, Johns Hopkins University School of Medicine, Baltimore, MD, Mar. 8, 2016
2. "Differential Expression of Transcription Factors in SIV-specific CD8+ T Cells at Week 5 and Week 20 After Vaccination with SIV239Δnef." James M. Billingsley, Premeela A. Rajakumar, **Yury V. Kuzmichev**, Nadine C. Salisch, Michelle Connole, R. Keith Reeves, and R. Paul Johnson. Poster session at the AIDS Vaccine 2011, Bangkok, Thailand, Sep. 12-15, 2011.
3. "Molecular Cloning and Characterization of NKG2D Ligands in Rhesus Macaques." **Yury V. Kuzmichev**, Henoch S. Hong, Rhona L. Glickman, Premeela A. Rajakumar, Gang Qiu, and R. Paul Johnson. Poster session at the 28th Annual Symposium on Nonhuman Primate Models for AIDS, New Orleans, LA, Oct. 19-22, 2010.
4. "Development of Real-Time PCR Assays for the Quantitation of Macaque Killer Immunoglobulin-like Receptors (KIR) Sequences." Emily B. Krulewitz, **Yury V. Kuzmichev**, Arnaud Colantonio, David Evans, Premeela A. Rajakumar, and R. Paul Johnson. Poster session at the 12th Annual Poster Session, New England Primate Research Center, Harvard Medical School, Southborough, MA, Aug. 11, 2010.
5. "Rational Design of Sequence-Specific Primers for Quantification of Natural Killer Cell Receptors in Rhesus Macaques." **Yury V. Kuzmichev**, R. Keith Reeves, and R. Paul Johnson. Oral Presentation given at the Undergraduate Research Colloquium. Pittsburg State University, Pittsburg, KS, Apt. 29, 2009. Poster session at the Fall 2008 Arkansas INBRE Research Conference, Fayetteville, AR, Nov. 7-8, 2008. Poster session at the 10th Annual Poster Session, New England Primate Research Center, Harvard Medical School, Southborough, MA, Aug. 5, 2008.

6. "ACS MoKanOk Local Section. Student Affiliates 2007-2008." **Yury V. Kuzmichev**, Anthony Truong, Carrie Nelson, Lindsay Nance, Blake Allen, and Khamis Siam. Poster session at the 10th Annual ChemLuminary Awards poster presentation at 236th American Chemical Society National Meeting & Exposition, Philadelphia, PA, Aug. 18-20, 2008.
7. "Resonance Assignment of Double-Stranded DNA using High-Field NMR Spectroscopy and its Applications in Determination of its Three-Dimensional Structure." **Yury V. Kuzmichev**, Kyle L. Brown, and Michael P. Stone. Poster session at the Fall 2007 Arkansas INBRE Research Conference, Fayetteville, AR, Nov. 9-10, 2007. Poster session at the 42nd Midwest Regional Meeting of the American Chemical Society, Kansas City, MO, Nov. 7-8, 2007.
8. "Coumarin Derivatives as Novel Antifungal Seed Treatments." **Yury V. Kuzmichev**, Nancy L. Brooker, Jacob C. Laas, and Robert R. Pavlis. Poster session presented at the 233rd American Chemical Society National Meeting & Exposition, Chicago, IL, Mar. 25-29, 2007.
9. "Thoracic Sympathectomy in Treatment of Complex Regional Pain Syndrome of Upper Limbs." Golubev V. **Kuzmichev. Y.**, G., Krupatkin A. I., Zeinalov V. T., Merkulov M. V., Kuzmichev V. Poster session at the 7th International Symposium on Sympathetic Surgery, Porto de Galinhas, Pernambuco, Brazil, Mar. 20-22, 2007.
10. "Synthesis of Coumarin Derivatives for Possible Use as Soybean Seed Treatment." Jacob C. Laas, Robert R. Pavlis, Nancy L. Brooker, and **Yury V. Kuzmichev**. Poster session at the Fall 2006 Arkansas INBRE Research Conference, Fayetteville, AR, Nov. 3-4, 2006. Poster session at the 41st Midwest Regional Meeting of the American Chemical Society, Quincy, IL, Oct. 25-27, 2006.

UNDERGRADUATE RESEARCH EXPERIENCE

Pittsburg State University, Department of Chemistry

*Research Assistant in the laboratories of Dr. James McAfee and
Dr. Irene Zegar*

Jan. – July 2009,

Jan. – May 2008

- Used fluorescence technique to investigate the binding of flavonoids to DNA
- Evaluated binding parameters including binding constants as well as determined cooperativity method in various flavonoid compounds
- Assisted with investigation of the proteins associated with the repressor complex

Pittsburg State University, Department of Biology

Research Assistant in the laboratory of Dr. David Gordon

Jan. – July 2009

- Modeled human population migration rates in the presence of variable factors
- Developed custom VBA codes for simplification of the calculations

Pittsburg State University, Department of Biology

Research Assistant in the laboratory of Dr. Nancy Brooker

Sept. 2006 – Apr. 2007

- Worked on the development of biopesticides for soybean seedling protection against early season fungal disease
- Designed and performed a variety of phytotoxicity experiments in order to better understand and describe the toxic effect of compounds on fungal growth

Pittsburg State University, Departments of Chemistry and Physics

Research Assistant in the laboratories of Dr. Khamis Siam and Dr. Robert Backes

Feb. 2005 – Feb. 2006

- Maintained and optimized a Beowulf Cluster (12 nodes) using Ubuntu and Open MPI
- Developed and refined Monte Carlo simulation describing the interaction of photons as they move through tissue (retina)
- Assisted with optimization of computational models of the spectral reflectance of human eye

TEACHING EXPERIENCE

Teaching Assistant

Graduate Immunology: The Immune Response – 260.717

Johns Hopkins Bloomberg School of Public Health

Mar. – May 2016

Biochemistry I Laboratory – CHEM*575*01

Pittsburg State University

Aug. – Dec. 2007

Tutor

English and Russian Language

Moscow Language Exchange

May – Aug. 2012, -13

General Chemistry I and II, Organic Chemistry I and II

Pittsburg State University

Jan. 2005 – May 2009

HONORS, AWARDS, AND FELLOWSHIPS

Pan American Society for Clinical Virology Travel Award	2016
<i>American Society for Microbiology</i>	
Master's Tuition Scholarship	2015 – 2016
<i>Johns Hopkins Bloomberg School of Public Health</i>	
Outstanding Senior	2009
<i>Pittsburg State University</i>	
Analytical Chemistry Award	2009
<i>Pittsburg State University</i>	
Undergraduate Research Colloquium – 1st Place	2009
<i>Pittsburg State University</i>	
International Certificate of Recognition	2009
<i>Pittsburg State University</i>	
International Advocate Scholarship	2005 – 2008
<i>Pittsburg State University</i>	
Undergraduate Russian Chemistry Scholarship	2004 – 2008
<i>Pittsburg State University</i>	
Certificate of Appreciation from the Dean of College of Arts & Sciences Student Advisory Committee	2009
<i>Pittsburg State University</i>	
Summer Pre-Baccalaureate Training Program Fellowship	2008
<i>Harvard Medical School, New England Primate Research Center, Division of Immunology</i>	
ACS ChemLuminary Award National Finalist	2008
<i>American Chemical Society 236th National Meeting and Exposition</i>	
Undergraduate Research Conference Ark-INBRE – 1st Place	2008
<i>Arkansas INBRE Research Conference</i>	

Golden Gorilla Award <i>Pittsburg State University</i>	2008
Biochemistry Award <i>Pittsburg State University</i>	2008
Chemistry Club Chemistry Award <i>Pittsburg State University</i>	2008
Schoene, Norberta Wachter Scholarship <i>Pittsburg State University</i>	2008
Summer Research Experience for Undergraduate Fellowship <i>Vanderbilt University, Department of Chemistry</i>	2007
Undergraduate Research Conference Ark-INBRE – 2nd Place <i>Arkansas INBRE Research Conference</i>	2007
Certificate of Appreciation Chemistry Club <i>Pittsburg State University</i>	2007
Undergraduate Research Conference Ark-INBRE – 3rd Place <i>Arkansas INBRE Research Conference</i>	2006
Gier, Leland J. And Margaret E. Biology Scholarship, Keller, Leland Endowment <i>Pittsburg State University</i>	2006
CRC Freshman Chemistry Achievement Award <i>Pittsburg State University</i>	2005

EXTRACURRICULAR AND LEADERSHIP EXPERIENCE

Student Outreach Resource Center (SOURCE), Johns Hopkins Bloomberg School of Public Health
2015-16 Cohort HIV Counseling and Testing Site Leader for Sisters Together and Reaching (STAR)

Oct. – May 2015

- Managed 9 students by assigning them to respective time slots, facilitated communication between respective teams, and increased overall efficiency of the site

**Generation Tomorrow, Johns Hopkins University –
Center for AIDS Research**

Oct. 2014 – Jun. 2016

HIV/HCV Counseling and Testing

- Conducted rapid HIV/HCV testing and counseling on more than 100 patients in various locations in Baltimore
- Assisted in the development of a HIV/HCV risk-reduction campaign for men of color focused on sexual behaviour

**Student Assembly, Johns Hopkins Bloomberg School of
Public Health**

Jun. 2015 – May 2016

Member-at-Large

- Presided on the Social Activities Committee (co-organized Fall Formal for 450 students), Community Affairs Committee (co-organized an STD awareness event), and University-wide Health Services Committee (oversight of the health agenda for the East Baltimore Campus)

**MMI Student Group, Johns Hopkins Bloomberg School of
Public Health**

Sept. 2014 – May 2016

Social Coordinator

- In charge of planning and organization of game nights for the MMI (Molecular Microbiology and Immunology) Student group

**International Injury Research Unit, Johns Hopkins
Bloomberg School of Public Health**

Oct. 2014

Russian Translator

- Assisted in communication between representatives from Russia and other countries during annual meeting

**Chemistry Club (ACS Student Affiliate Chapter)
Pittsburg State University**

Sept. 2006 – May 2009

President

- Led the club to be nominated for a National American Chemical Society (ACS) Award ChemLuminary 2008 MOKANOK (Missouri, Kansas, Oklahoma) local section for outstanding celebration of Earth Day
- Responsible for leading a team of 20 during National Chemistry Week 2008 where the club was responsible for organization of Chemistry Magic Shows for Elementary and Middle Schools in the surrounding areas (4 TV appearances in 7 days)
- Coordinated the establishment of annual Chemistry Club Scholarship to high school seniors interested in chemistry

- Expanded the club membership from five to 50 active members
- Organization of various fund-raisers, Garage Sale, a highway cleanup, ChemPardy, adopt a family for Thanksgiving and Christmas, celebration of Mole Day, Earth Day, Science Day, and the Chemistry Olympiad

Admissions Office, Pittsburg State University

Aug. 2008 – Feb. 2009

Student Ambassador

- Responsibilities included serving one hour a week in the Admissions Office, visiting with prospective students, taking visitors to campus appointments, giving campus tours, and assisting with various on-campus events, such as “Jungle Day”

**Student Advisory Council, College of Arts and Sciences,
Pittsburg State University**

Sept. 2008 – May 2009

Departmental Representative

- Promoted interests of the Chemistry Department (e.g. increase in the number of tutors for midterm and final examinations)
- Acted as a liaison between the Department of Chemistry and the Dean of College of Arts and Sciences

**Student Government Association,
Pittsburg State University**

Jan. – May 2008

Senator

- Promoted the Student Government Association and its projects and initiatives at Pittsburg State University
- Assisted on various projects with the Student Body Board of Elections and Academic Affairs Committee (e.g. nomination and selection of outstanding faculty)

Crimson Club, Pittsburg State University

Aug. 2006 – May 2007

Member

- Represented the student body and the Office of the President at such events as the President’s Society Dinner, receptions for international visitors, major donors, distinguished faculty, and assisted during campus tours by state and federal government officials

**International Student Association
Pittsburg State University**

Aug. 2005 – May 2009

International Advocate

- Assisted during orientations for new international students, organized cultural presentations and food exchanges

Pre-Med Club, Pittsburg State University*Sept. 2004 – May 2009**Member*

- Contributed to the “Pre-Med Buddy System” by advising students on class schedules, shadowing opportunities, and research tracks, participated in various fundraisers

Biology Club, Pittsburg State University*Sept. 2004 – May 2009**Member*

- Designed a beta version of the club website

OTHER EMPLOYMENT**MEDEX LLC***Moscow, Russia**Project Manager and Marketing Assistant**Mar. 2012 – Aug. 2014*

- In charge of coordination of educational seminars for physicians on major products from the company’s portfolio including SWI-STO II Iontophoresis and implants for treating erectile dysfunction and urinary incontinence (AMS 800, AMS 700MS)
- Coordinated development and deployment of a web application for doctor-patient interactions and appointment scheduling
- Revised and updates advertising campaigns for company’s products (Human Energy 107, Maxim, Odoban, DRY24, and surgical equipment)

VOLUNTEERING AND OTHER ACTIVITIES**VOLUNTEERING****Moscow Research Institute MONIKI***Moscow, Russia**Preventive Medicine**Aug. 2012 – Aug. 2013*

- Assisted in organization of health screening booths offering cholesterol, blood glucose level, and blood pressure testing for over 2000 patients
- Coordinated efforts to encourage patients to keep a list of their current medications within a medicine bottle for easy access in case of an emergency through promoting the Vial of Life (adapted for use in Russia)

Marlborough Hospital*Marlborough, MA**Assistant in the Transportation Department**Oct. 2011 – Mar. 2012*

- Transported patients from their rooms to various treatment areas and back
- Delivered supplies and materials from central supply to assigned areas

Meals on Wheels, Mt. Carmel Regional Medical Center Dietary Department*Pittsburg, KS**Jan. – May 2009**Driver/Deliverer*

- Delivered an average of 150 meals during each month to elderly and disabled persons

Moscow Research Institute MONIKI*Moscow, Russia**Critical Care Medicine**May – Aug. 2004*

- Comforted patients and their family members in waiting rooms
- Directed incoming patients to appropriate areas (triage, registration, recreation area)
- Performed suture removal and participated in the change of medical dressings

MEDICAL SHADOWING EXPERIENCE**Moscow Research Institute MONIKI***Moscow, Russia*

- Department of Critical Care Medicine

May – Aug. 2013

- Department of Thoracic Surgery

*May – Aug. 2006***Carolinas Medical Center and Heineman Medical Research Center***Charlotte, NC*

- Department of Thoracic and Cardiovascular Surgery

*May 2010***SLUCare, Des Peres Medical Arts Pavilion***St. Louis, MO*

- Division of Cosmetic and Laser Surgery

*Aug. 2007***Newton Memorial Hospital and Neurologic Arts Associated LLC***Newton, NJ*

- Department of Neurology

*Dec. 2005 – Jan. 2006***Faith Regional Health Services***Norfolk, NE*

- Department of Cardiovascular and Thoracic Surgery

Dec. 2004 – Jan. 2005

PROFESSIONAL MEMBERSHIPS AND HONOR SOCIETIES

- American Society for Microbiology, American Chemical Society, Omicron Delta Kappa, Phi Kappa Phi, TriBeta Biological Honor Society

Last Revised: April 10, 2016